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An Ultrastructural Study of Fresh and Ensiled Alfalfa Using the Light and Transmission Electron Microscope

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AN ULTRASTRUCTURAL STUDY OF FRESH AND ENSILED ALFALFA USING THE LIGHT AND TRANSMISSION ELECTRON MICROSCOPE

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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KEY TO ABBREVIATIONS USED IN FIGURES

C	Chloroplast
Ca	Cambium
Ch	Chlorenchyma
Cl	Collenchyma
CW	Cell Wall
Cy	Cytoplasm
DCy	Disrupted Cytoplasm
DP	Disrupted Pith
E	Epidermis
G	Granum
IA	Intracellular Air Space
L	Lipid
La	Labyrinth
Lt	Lignified thickenings of xylem vessels
M	Mitochondria
Mb	Microbody
Me	Membrane
ML	Middle Lamella
Ms	Meristematic
Nu	Nucleus
P	Pith
Pd	Plasmodesmata
Ph	Phloem
PM	Plasma Membrane
R	Ribosomes
RER	Rough Endoplasmic Reticulum
S	Starch
SER	Smooth Endoplasmic Reticulum
TC	Transfer Cells
U	Unidentified Cellular Contents
V	Vacuole
VB	Vascular Bundle
X	Xylem
◄	Indicates areas at interest in the micrograph which are explained in the Results and Discussion Section

ABSTRACT

Late bud alfalfa was sampled prior to cutting and after 17 and 51 days of ensiling. Leaf and stem tissue were separated and immediately placed in 2.5% glutaraldehyde followed by 1% osmium textroxide for fixation. Following acetone dehydration, samples were embedded in Spurr plastic for examination under the light (LM) and transmission electron (TEM) microscopes. Light microscopy was used to differentiate cell types and tissues, e.g. epidermis, chlorenchyma, phloem, xylem, and pith. Iodine staining (LM) showed starch localization in chlorenchyma cells. Transmission electron microscopy analysis showed starch localized in chloroplasts of chlorenchyma. Leaf cells averaged nine chloroplasts per cell section and eight starch granules per chloroplast section. Stem cells averaged four chloroplasts per cell section and three starch granules per chloroplast section. After ensiling, only remnants of starch were observed. Lipid was visible as coalesced droplet in ensiled tissue, but was incorporated in the intracellular structures of fresh tissue e.g. membranes. Intracellular material of fresh tissue was localized on the inside periphery of the cell. Vacuoles occupied the central portion. This integrity was lost in the ensiled samples. Light microscopy and TEM showed cell wall breakage in chlorenchyma and pith cells, but cell walls from vascular bundle and epidermal cells remained intact. Some midele lamella disintegration occurred in all cellular regions. Results indicate the LM and TEM could be useful as tools in forage research.

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INTRODUCTION

Silage is: "The feedstuff resulting from the anaerobic preservation of moist forage or other feedstuffs by the formation and/or additions of acid" (25).

Silage offers several advantages over hay for increased usage by farmers. Reduced harvesting losses and less chance of weather damage at harvesting improve the quality of the forage. Since silage involves harvesting the whole plant, the maximum amount of nutrients per acre are harvested. Silages are an excellent roughage source and are easily handled by mechanical means. Silage can also increase land usage since double or triple cropping is possible.

Much research has been designed to study ways to control and improve the ensiling process. Two reviews on silage, Watson and Nash (35) and McCullough et al. (25), both concluded that in silage research no major breakthrough has occurred since the 1939 silage review by Watson (34). Research has been able to improve silage making techniques and give better understanding of the techniques, but it is still not possible to totally control the process of fermentation in the silo. The question posed by McCullough et al. in 1978 was "Where do we go from here?" (25).

Previous research has been concerned with methods of harvesting, ensiling, and management techniques which give the most desirable silage. Some of the previous research areas have dealt with the incorporation of additives, improvement of silos, stage of maturity best for harvesting, length of forage chop, and different ways

to feed silage so as to increase the feeding value to livestock.

The research for this thesis involves the use of the transmission electron microscope (TEM) and the light microscope (LM) to determine structural changes occurring at the ultrastructural level before and during the ensiling process.

LITERATURE REVIEW

Early Chemical Analysis Research

The analysis of forage fiber dates back over 150 years (30). Fiber was one of the first plant components to be analyzed because early researchers believed the fiber fraction was the indigestible part of foods and feedstuffs (30). Crude fiber is defined currently as that fraction of the cell wall which contains insoluble and non-digestible carbohydrates and carbohydrate complexes, i.e., cellulose and lignin (14).

One of the first systems developed for plant analysis was the Weende system of proximate analysis. It is a system designed to analyze feedstuffs for crude fiber, dry matter, ether extract, crude protein, ash, and nitrogen-free extract (16). The Weende system is of German origin, but its originator is unknown (28).

Einhof, in 1809, used a technique involving forage tissue masceration followed by extraction with water to determine crude fiber (32). Einhof also used a technique involving a sequential extraction of a forage with alcohol, dilute acid, and dilute alkali (28, 32). Einhof's water extraction values are similar to Van Soest's neutral detergent fiber values for the same feedstuffs (32). Gorham, in 1820, used an alkali extraction of forage tissue to obtain values comparable to crude fiber values determined by the Weende system of proximate analysis (32).

The method of forage nutritive evaluation during Einhof's time was the use of hay equivalents, since fiber was assumed to be an

indigestible fraction (28, 32). Haubner, in 1855, by means of a digestion trial, found that portions of the fiber fraction were digestible (28, 32). Henneberg and Stohmann demonstrated in 1860 that fiber was partially digestible (28, 32). Because of the work by Haubner, and Henneberg and Stohmann, the hay equivalent system for the nutritive evaluation of feedstuffs was abandoned (28, 31).

The purpose of forage analysis is to estimate nutritive value by chemical means. The current chemical system of analysis, the Weende system of proximate analysis, has been used without modification since 1860 (30). Researchers since 1860 have been forced to use the inadequacies of the system, in particular the inadequacies of the crude fiber determination. The problem with the crude fiber determination is that it is not always related to quality of a forage and the crude fiber value of a given feedstuff is sometimes more digestible than the nitrogen-free extract, which by definition, is the highly digestible carbohydrate fraction (30). The continued use of crude fiber is a result of a lack of understanding with respect to what crude fiber represents biochemically and to the fact that a better system has not been developed to replace crude fiber. This is changing due to more recent research by Van Soest.

Van Soest's Methods for the Chemical Analysis of Forages

The Van Soest method of forage analysis divides plant dry matter into two fractions: cell wall constituents (CWC) and cell solubles (30, 31, 32). The cell solubles are assumed to be totally digested by the animal. Cell solubles represent approximately 40%

and 60% of the dry matter in grasses and legumes, respectively, and approximately 90% of the dry matter in concentrates (31). The cell wall is divided into its constituents by the use of detergents (17, 30). A neutral detergent solution is used for extraction and the residue remaining is the CWC, i.e., hemicellulose, cellulose, lignin, and ash. Subjecting the CWC to extraction in acid detergent solution solubilizes hemicellulose leaving a fraction known as acid detergent fiber (ADF) which contains cellulose, lignin, and ash. Lignin is determined on the ADF residue by means of permanganate oxidation or the use of 72% H_2SO_4. Cellulose and ash remain after this step. Cellulose is determined as that weight lost due to combustion in an ashing oven.

The cell wall is the limiting factor in forage utilization (20, 28, 32). Several factors affect cell wall digestibility and ultimately dry matter digestibility. These factors include the plant species, the animal's voluntary intake of a forage, proportion of forage to concentrate, and rate of passage through the gastrointestional tract (29, 32). Current chemical methods of analysis are unable to accurately estimate digestibility since chemical analysis techniques do not separate all the digestible fractions in the cell wall from the indigestible fractions (30, 32).

Van Soest's work on chemical analysis of cell walls and on in vitro digestibility involves forage consumption by ruminants and how it is regulated by certain chemical factors. One of these factors, voluntary intake, is negatively correlated to the cell

wall. This is because the cell wall is the plant skeleton which contains the plant's nutrients and thus, determines the plant's caloric density (32). Similarily, the subsequent intake of a forage is positively correlated to rate of passage because the cell wall is negatively correlated to dry matter digestibility and thus, it decreases the rate of passage when compared to cell solubles (28, 29, 32). The cell wall is composed of cellulose, lignin, cuticle, hemicellulose, ash, and pectins (32). Pectins are completely digestible by the ruminant and are extracted by neutral detergent solution. Cellulose and hemicellulose are partially digestible by the ruminant and ash, lignin, and cuticle are not digestible. The cuticle is extracted in the lignin fraction. Table 1 further explains the relationships between Van Soest's detergent fractions (32). One means of measuring digestibility is by in vitro digestibility techniques. Van Soest's research has shown in vitro digestibility at 6 h is correlated to dry matter intake and at 36 h is correlated to in vivo digestibility (32).

Van Soest's work has introduced methods to estimate a feedstuff's value with the use of new chemical analyses (17, 28, 29, 30, 31, 32). Van Soest has shown that rate of digestion is dependent on the feedstuff's chemical and physical composition which affect rate of passage and level of intake; variables set by the animal but dependent on the feedstuff (32). Since the cell wall constituents are the slowest digesting fraction, current research is needed to determine ways to increase the cell wall constituents' digestibility (32).

TABLE 1. Correlations between composition of forage, voluntary intake, and digestibility (32).

Component	Number of forages	Voluntary intake	Dry matter digestibility
Cell wall	14[a]	-.66	
	77[b]	-.56	-.32
	187[c]	-.77	-.45
	83[d]	-.65	-.65
Acid detergent fiber	14[a]	-.13	
	77[b]	-.31	-.74
	187[c]	-.64	-.75
	83[d]	-.53	-.74
Lignin	14[a]	-.09	
	77[b]	+.21	-.64
	187[c]	-.08	-.61
	83[d]	-.13	-.52
Cellulose	14[a]	-.14	
	187[c]	-.75	-.56
	83[d]	-.59	
Dry matter digestibility	77[b]	+.08	
(in vivo)	187[c]	+.44	
	83[d]	+.66	
Dry matter digestibility (in vivo)	187[c]	+.47	+.80

[a]Brahmakshatriya, R. D. 1971. A comparison of physical methods of forage evaluation. Ph.D. Thesis. Univ. of Minnesota.

[b]Johnson, R. R., and B. A. Dehority. 1968. A comparison of several laboratory techniques to predict digestibility and intake of forages. J. Anim. Sci. 27:1738-1742.

[c]Mertens, D. R. 1973. Application at theoretical mathematical models to cell wall digestion and forage intake in ruminants. Ph.D. Thesis. Cornell Univ., Ithaca, NY.

[d]Van Soest, P. J. 1965. Symposium on factors influencing the voluntary intake of herbage of ruminants: voluntary intake in relation to chemical composition and digestibility. J. Anim. Sci. 24:834.

Other Tools and Methods Used in the Analysis of Feedstuffs

In addition to chemical means of forage analysis and evaluation, other methods are currently being tested. Infrared reflectance spectroscopy (IR) can be a rapid accurate means for measuring nutrients in forages, grains, and mixtures of the two (27). Current research with IR is concentrated on improving the instrumentation currently in use by developing an instrument capable of selecting the desired set of wavelengths needed for a particular sample, and after the sample is read analyzing the data by computer. Although the IR is both rapid and accurate, the knowledge of calibrating the machine for all feedstuffs now fed is not known. Since the IR spectra for each constituent, i.e., amino acids and minerals is different, analyzing feedstuffs for constituents by IR constitutes a complex problem which is part of the research Shenk and co-workers (27) are currently involved in. The IR must be recalibrated for each different feedstuff analyzed; i.e., the IR will read alfalfa proteins from fresh plants after calibration, but must be recalibrated for alfalfa proteins from field dried plants. The IR has been used also in predicting animal digestibility responses to forages.

The use of microscopic techniques is another means of understanding feedstuffs and fiber biochemistry. These can be divided into three categories: light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

Light microscopy has a magnification range from 20x to 1000x. Its resolution is approximately 200 mμ. The purpose of its use is

to get an overview of the specimen or section. This is necessary to identify ultrastructure when viewed at a larger magnification. Other uses are special techniques in lighting such as ultraviolet irradiation, or phase contrast, etc.

When SEM is used, a survey of the specimen's exterior is made. Usually low magnifications of < 500x to 10,000x are sufficient and resolution is 25 mμ or smaller (2). This tool is used to obtain an external overview, and it is very useful for observing the integrity and structure of the exterior and changes which can occur after treatments.

Transmission electron microscopy is used when an interior section is needed. It helps to show internal structures with magnifications of 1000x to 100,000x. Resolution is generally about 0.24 to 1.0 mμ (2).

Microscopic Analysis of Plant Anatomy With Respect to Differences and Digestibilities of Microanatomy

Microscopy is a valuable tool for understanding cell ultrastructure. Light microscopy has been used to show anatomical differences in grass types (warm season vs cool season) and to classify grasses with respect to digestibilities and environmental growth conditions (4, 5, 6, 7, 8, 9, 10, 13, 18, 26). Thus, microscopic analysis is suited for plant breeding and selection studies due to small sample needed and it is a rapid method (2, 26). Microanatomical differences have been found in warm season grasses by LM, SEM, and TEM (4, 6, 7, 9). One difference found in the grasses studied; Coastal and Coastcross-1 bermudagrass, Pensacola bahiagrass, and

Kentucky-31 tall fescue (a cool season grass); was the quantity and location of the lignin (4, 6, 7, 9). Lignin was reported in all the grasses, and found in the tissue which separates xylem and phloem in all first order vascular bundles (6, 7). However, bermudagrass possessed a highly lignified inner bundle sheath whereas, Pensacola bahiagrass was only partially lignified (6, 7). Second order bundles were found to have a single nonlignified bundle sheath (6, 7). In tall fescue the vascular bundles were double sheathed, with the inner sheath slightly lignified (6, 7). Results indicate these microanatomical differences may be factors causing differences in digestion times and rate of digestion appears to be due to cell wall organization or tissue degradability differences (4, 6, 7, 9).

Rumen Microflora Interactions With Rumen Cells and Feedstuffs

Another area of microscopic research on forages deals with rumen microorganisms and their interrelationships with feedstuffs in the rumen (1, 15, 21, 22, 24). Monson et al. (24), were able to show by microscopy that rumen microorganisms did not enter the plant through cuticles of stomates during rumen digestion but through a break or a cut in the leaf tissue. This conclusion was further supported in some research by Latham et al., who described the mode of adhesion of Ruminococcus flavefaciens, a rumen cellulolytic bacterium, to cotton cellulose and to leaf cell walls of Lolium perenne L. (perennial rye grass) with the use of TEM (21). Bacteria entered into the plant in areas where the exterior surfaces were damaged because of the inability of the bacteria to adhere to

an undamaged cell wall. This is important because if there is a way to damage more cell walls during harvesting, cell contents could be more readily available for digestion by the ruminant (21, 24).

The adhesion of gram-positive bacteria to stratified squamous epithelium cell surfaces in the reticulo-rumen of cattle was studied with SEM to get general overviews of action and with TEM to study the actual relationship of the adhesion by the bacteria (22). Stratified squamous epithelium cells in the cattle's reticulo-rumen supports a large population of stationary adhering gram-positive bacteria. This adhesion provides protection and serves as a food source since the contents of the reticulo-rumen are continually coming into contact with the bacteria. There is a specificity for stratified squamous epithelium cells by gram-positive bacteria and a synergistic effect between the epithelial cells and the bacteria. The bacteria feed on the reticulo-rumen contents and help the epithelial tissue slough its dead superficial cells.

Cheng et al. (15), using TEM, studied changes which occurred in rumen bacterial cell envelope morphology under various in vivo conditions. Results show that the majority of rumen bacteria have more surface layers and more extensive capules than the same bacteria types found outside the rumen. This was interpreted as an adaptation to a continual changing rumen environment.

One rumen microbe identified from gram-stained smears of rumen microflora by SEM and TEM, is a small, filamentous, branching,

gram-positive microorganism that degrades tissues in forages during in vitro and in vivo incubation with rumen fluid (1). Round bodies, attached to hyphae, resemble a sporulation pattern reported for Micromenospora. Filaments, rods, and cocci forms of this microbe have been reported degrading rigid forage cell walls and lignified, thick-walled sclerenchymal cells. This microbe is located a small distance away from the degraded zones and this suggests the action of extracellular enzymes. This microbe degrades lignified tissue. Since the ruminant is incapable of lignin digestion itself, nutrients bounded by lignified cell walls and bonded to lignin complexes are not utilizable unless microorganisms in the rumen can release the nutrients by breaking down the lignin-nutrient complex. This microbe, by lignin digestion, is able to make more nutrients available for digestion by ruminants and consequently, aids in an important and unique function of the rumen microflora.

Studies on Feedstuff Digestibilities in the Rumen by the Rumen Microflora

Akin (2) states that "microscopic techniques could be used for rapid comparisons of the rate and extent of fiber digestion. Those techniques could be applied to forage breeding programs to improve forage quality or in programs to evaluate the influence of environment or management practices on cell wall digestibility". His research and the research of others gives evidence of a specific order of digestion by rumen microflora of a feedstuff (7, 8, 10, 12, 13, 18, 19). Since forages can't be entered by bacteria through

the cuticle or stomates (21, 24), in order for digestion to occur, the microbes must enter at a break or create a break in the cuticle. Barzle and Harbers (13), using SEM, found the cuticle and epidermis of Medicago sativa (alfalfa) to be sloughed after 12 h in vivo digestion in the rumen for leaf and 24 h for stem tissue. Akin (10) observed a preferential splitting of the cuticle of coastal bermudagrass by a bacterial attack on the underlying epidermal cells. The cuticle was pealed back and remained undigested. Once the bacteria entered either the leaf or stem they degraded mesophyll and phloem tissues rapidly and before any other tissues (3, 7, 8, 10, 13). Akin (10) found that mesophyll and phloem cell walls degraded rapidly with what is believed to be extracellular enzymes of bacterial orgin. Bundlesheath cells and epidermal cells were degraded last leaving only the lignified vascular tissues remaining (7, 10). Akin (10) noted bacterial attachment to the bundle sheath and epidermal cells resulting in degradation of those cells. Lignified cells remained undigested after 72 h incubation with rumen microflora (3, 4, 13, 18).

In addition to plant cell wall digestion, Akin and Burdick (8) studied the degradation of starch in bundle sheath cells by rumen microbes after 6 h in vitro incubation in buffered rumen fluid. Leaf sections from Coastal bermudagrass, Pensacola bahiagrass, and Pangola digitgrass were used. Iodine-potassium-iodine (I-K-I) stained sections viewed with LM showed most of the starch to be present in the chloroplasts of the parenchymal bundle sheath cells.

Little or no starch was noted in mesophyll cells, which were more rapidly degraded than the parenchymal bundle sheath cells in all species. TEM showed starch to be degraded after the cell walls of the thick, laminated parenchyma sheaths were disrupted or degraded by the rumen bacteria. Results indicate that a lot of starch remains unavailable to the ruminant because of the starch containing parenchymal bundle sheath cells in warm season forages.

Barton and Akin (12) studied the effects of delignification on rumen digestibility of cell walls from Coastal bermudagrass and Kentucky-31 tall fescue. Ground forage samples were delignified with potassium permanganate in an acetic acid buffer then subjected to in vitro incubation for 1, 2, 4, 6, 24, and 48 h in buffered rumen fluid. The sclerenchymal tissue showed more susceptibility to the permanganate treatment than the inner bundle sheaths. All tissues were found to be more readily digested with the exception of the vascular bundle which was not digested. The conclusions reached indicate a hinderance of digestion not only due to lignin, but to structural carbohydrates as well. Also, Akin et al. (5), studied leaf sections from Coastal bermudagrass and Kentucky-31 tall fescue which had been extracted with neutral detergent reagent for 60 min and with acid detergent reagents for 10, 30, or 60 min. Control samples were extracted in water. Acid detergent extraction for 10 and 39 min removed the same tissues that were shown to be digested during rumen microbial digestion. The 60 min acid detergent residues were mainly rigid lignified tissues. The Coastal bermudagrass

tissues were extracted slower and to a less extent when compared to the tall fescue. The acid detergent residue appeared to contain all the lignified cell wall constituents. These studies using microscopy help in further explaining the relationships between chemical composition and rumen microbial digestion noted by Van Soest (28).

The majority of microscopic research on forages has compared forage digestion by rumen microflora and ultrastructure of forages. Ward et al. (33), showed why the utilization of calcium from alfalfa was consistently less than that present in the plant. They studied samples, using SEM, from nylon bags subjected to 24 and 48 h of in vivo rumen digestion. They also viewed feces from cows fed high alfalfa diets. Both sets of samples showed crystals present on the parenchymatous sheaths around the vascular bundles and isolation of these crystals using microcrystal separation techniques revealed the crystals as mostly calcium oxalate with some potassium oxalate present. Under SEM the crystals were contained within a capsule and this prevented the utilization of the calcium found in the crystals but not tied up in oxalate bonds. These crystals represent 20 to 30% of the calcium in alfalfa. They recommend a 25 to 50% discount of the calcium in alfalfa for ration formulation purposes.

The preceeding literature review expresses some of the inadequacies of the Weende system of proximate analysis, citing examples where current research methods for fiber analysis developed by Van Soest would be more accurate means of analysis. It also covers some

of the previous microscopic research in the area of forages. Since a chemical analysis is not completely indicative of the ultrastructure of a feedstuff and since more research is needed to answer the existing questions on forages and how they can be improved as a feedstuff for livestock, the use of microscopy should be employed along with chemical analysis as research tools for forage studies. Previous research has shown that microscopic analysis can be useful in forage research. Consequently, this thesis research was designed to make a comparative study of compositional changes occurring during the ensiling process. Medicago sativa (alfalfa) was used at the late bud state of maturity and the fresh alfalfa was compared with the same alfalfa after 17 and 51 days of ensiling. The tools for analysis were TEM, LM, and chemical composition. Silage was studied due to a lack of silage microscopic research and because silage is an important feed of today's dairy cattle.

MATERIALS AND METHODS

Stems and leaves from late bud alfalfa were sampled at 1430 h on June 5, 1978. Samples for electron microscope (TEM) and light microscope (LM) examination were placed immediately into 2.5% glutaraldehyde at 3 C (see appendix). Samples for compositional analysis were put into plastic bags, sealed, labeled, and frozen at -20 C. Dry matter was determined the day of sampling. The same alfalfa stand was then cut and ensiled in a concrete bunker silo. The silage was sampled at 17 and 51 days with a core sampler. Sample preparation procedures were repeated for TEM, LM, compositional, and dry matter analysis.

Both fresh and ensiled samples for TEM and LM examination were embedded in the following manner in the laboratory. The fresh alfalfa was cut into approximately 3 mm pieces after wetting with freshly prepared 2.5% glutaraldehyde at 3 C. Similarly, the alfalfa silage was put into freshly prepared 2.5% glutaraldehyde at 3 C then removed to separate and cut leaves and stems for embedment. After cutting samples were placed in 2.5% glutaraldehyde at 3 C for 30 min. Vials were then drained of glutaraldehyde and 1% OsO_4 at 3 C (see appendix) was added. The samples remained in OsO_4 for 30 min.

Samples were then dehydrated with a series of acetone washes. The samples were first rinsed, then washed for 5 min, then for 15 min in 25% acetone. Samples were washed next in 50% acetone at 3 C for 30 min, drained and stored in clean 50% acetone for 69 h at 3 C. Next samples were washed in 75% acetone at room temperature for 15

min. The final acetone wash was in 100% acetone at room temperature for 20 min, then 100% acetone for 60 min. After this last wash samples were put into freshly prepared Spurr plastic (see appendix) to allow for the replacement of acetone with plastic in the tissue. The plastic and samples were stirred continuously for 15 min, then occasionally for 105 min and then heated in a drying oven at 57 C to remove the residual acetone. Following infiltration with Spurr, samples were embedded in fresh plastic. Thirty leaf and thirty stem samples were placed into individual Micromolds that were previously filled with Spurr plastic. The plastic was polymerized in an oven at 70 C for 9.5 h.

After the samples had been polymerized in plastic, they were prepared for viewing under the TEM and LM. To prepare the samples for viewing under the TEM the blocks were trimmed using a disecting microscope with an unused single edge razor blade. The trimmed blocks were put into a Porter and Blum MT-II ultramicrotome and sections of approximately 60-150 Angstroms were cut with a glass knife made with an LKB 7800B knife maker. The water trough, used for catching sections after they were cut, was made by melting wax and allowing it to dry on the glass knife about 5 mm from the cutting edge. The sections were picked up from the distilled water in the trough with a 200 mesh copper grid. The shiny side of the grid was always used and to prepare the grid for use it was placed on top of a rubber stopper and scrapped with the handle of the TEM tweezers, specialized for TEM work because of their fine point tips. Since

this caused the grid to curl, it was flattened by rubbing it with a test tube bottom. This procedure helped to reduce the static charge and allowed the grid to pick up the sections better.

Once the sections were picked up on the grid, the grid was placed section side down into a droplet of 2% uranyl acetate (see appendix). The grid was stained for 1 h and then removed and rinsed by careful vertical dunking five times each into four successive beakers of distilled water. After the last dunking the grid was placed face down into 0.5% lead citrate stain (see appendix) for 1 min. The grid was then removed and rinsed by careful vertical dunking into distilled water that had been boiled to remove CO_2 and then cooled. Each grid was then placed section side up on tissue paper and allowed to dry. After drying the grid was stored in plastic grid holders until examination under the TEM.

When sections were ready for examination under the TEM, they were put into a copper resolution specimen holder. The same holder was used throughout the TEM analysis. The grid was then put into the TEM and viewed with a spot size of 2, 5, or 10 in a Hitachi, Model HU-12 transmission electron microscope. Before the grids were put into the TEM the electron beam was aligned and the condenser lenses were stacked using common beam alignment and lens stacking procedures. Liquid nitrogen was used to cool the microscope in the critical areas when pictures were taken to prevent plastic melting contamination and consequent distortion due to the beam. Photographs were taken using 8.3 x 9.8 cm film (Dupont No.

7060-57-3) in place in the TEM camera. The chloroplast and starch granule counts were made by counting and recording starch granules seen in each chloroplast section and chloroplasts seen per cell section while viewing the sections under the TEM.

For LM, sections were cut in the same manner as for TEM except the sections cut were approximately 0.1 to 0.5 μ thick. They were then removed from the distilled water in the trough with TEM tweezers or with the section brush and put into a drop of cool distilled water on a glass microscope slide. The slide was then heated on a hot plate set on low to evaporate the water and melt the plastic to the slide. Slides were viewed under a Nikon LM Model M-35S, equipped with a Nikon camera (No. 73778, 1.25x) and with a Nikon photograph extension (No. 46719). To determine the starch present in the cells, slides were stained with Gram's iodine by putting a cover slip over the sections and a drop of the iodine next to the cover slip. By capillary attraction the iodine was drawn between the slide and cover slip. The slide was then ready for viewing under regular occular. Black and white photographs were taken of the sections with the in place Nikon camera.

Negatives from the TEM camera were developed in a special dark-room used solely for TEM negatives. The solutions used for development were Kodak D-19 developer for 5 min, a 27% acetic acid stop bath for 30 sec, Kodak fixer for 5 min and Kodak hypo clearing agent for 5 min followed by a 15 min continuously changing water rinse.

Photographs taken with the Nikon LM camera were taken on 135 mm

Kodak black and white 125 ASA Plus-X Pan film. This film was developed using Kodak D-76 developer for 5 min, a 27% acetic acid stop bath for 30 sec, Kodak fixer for 5 min, Kodak hypo clearing agent for 5 min, continuous changing water rinse for 15 min and a final rinse in photoflo.

Both TEM and LM negatives were used to make prints or enlargements on Kodak polycontrast rapid single wt F, 20.3 x 25.5 cm paper. This paper was exposed to the negative using a Durst D-6 enlarger and developed using Kodak Dektol developer until contrast was right, then placed in a 27% acetic acid stop bath for 30 sec, Kodak fixer for 5 min, and Kodak hypo clearing agent for 5 min followed with a 30 min continuously changing water rinse. The prints were then rolled dry with rollers as they lay on blotter paper and then dried face up on a Japo Auto Dryer, type S-3.

Photography for the photographs incorporated in the thesis was done by making the correct sizes and enlargements needed using the previously described procedure. These prints were then fastened to heavy, smooth poster board using Seal MT5, a permanent dry mounting tissue. The Seal tissue was tacked to the back of the pictures with a Sealector tacking iron. Excess tissue was trimmed off. The pictures were then put on to a piece of 21.5 x 28 cm posterboard with the tissue in between pictures and posterboards. This was put into a Technal Dry Mount Press at 104 C and held for 45 sec, then held flat by placing heavy books over the pictures and posterboard until they cooled. The pictures, once fastened to the posterboard,

were labeled and μ distances were marked using Ted Pella Dual-color code dry letter sheet.

To determine μ markers, the original magnification was multiplied by the photographic enlargement magnitude. This number was then divided by 1,000 and the resulting number in mm was equal to 1 μ (Equation 1).

Equation 1

$$\frac{(\text{microscope magnification}) \times (\text{photographic enlargement magnitude})}{1{,}000} = x \text{ mm} = 1\ \mu$$

Once the μ was determined in measures of mm, the size of line could be determined to fit the picture size.

After labeling, the plate was photographed using a Polaroid MP-4 land camera. Sylvania flood lights were used for illumination. Negatives of the plates were made using Polaroid positive negative 10.2 x 12.7 cm Type PN55 film. The negatives were developed for 25 sec by the chemicals enclosed in the pack holding the exposed negative. Each negative was then removed from its pack and placed in sodium sulfite for 3 min, followed by a 15 min water wash, which was followed by a photo flow rinse. The negatives were then used to expose Ecktamatic single wt paper of 21.5 x 28 cm. The Ecktamatic paper was then developed using the same chemicals used with the Kodak polycontrast rapid paper. This was the photograph used in the thesis; the dimensions being the same as the old council tree bond used to type the thesis text.

Presentation slides were taken on Ektrachrome 160 with F22 at

one-quarter sec and Kodachrome Professional Type A with F16 at 1 sec.

RESULTS AND DISCUSSION

One of the main objectives of forage research is to increase the availability of nutrients in the forage to livestock. Silage involves preservation of the forage by fermentation. Previous silage research has been studied using chemical analysis. This thesis research was undertaken to show the structure of Medicago sativa (alfalfa) before and after ensiling using the light and transmission electron microscopes as the tools of analysis. Cell ultrastructure and cell walls were observed from different cell types of the alfalfa leaf and stem using fresh tissue and tissue after 17 and 51 days ensiled as samples. A chemical analysis of the forage was also made for use as a comparison to previous research.

Since one objective of this research is to determine the usefulness of microscopy as a tool for analysis, some aspects of microscopy must be considered.

When viewing micrographs of tissue sections using transmission electron microscopy (TEM) and light microscopy (LM) respect must be given to the limited amount of information they contain and thus, interpretations of the micrographs must be made carefully.

In this research a typical alfalfa plant cell was approximated at 10μ in length. When one cell was examined using LM a 0.5μ thick cross-section was viewed. Thus, the micrograph taken contained approximately 5% of the total cell's information. Similarly, using TEM, a 100 nm section was viewed and this represented 0.01% of the

cell's information. Most of the samples put into plastic for sectioning were 2.5 mm^3. Each sample contained approximately fifty billion cells. Thus, since one transmission electron micrograph represents 0.01% of a cell's information, and there are fifty billion cells per sample, and each sample represents a small fraction of the total cellular mass in a field; the conclusions drawn from transmission electron micrographs must be supported with more analytical methods. Thus, chemical analysis of the forage was done, and no statistical analyses were performed due to the small sample size used.

The light microscope was used in this research to obtain an overview of the cellular ultrastructure. The light microscope allows many different cell types in a section to be photographed. The transmission electron microscope was used for cell detail because of its high magnification since only a few cells can be photographed on one negative.

Some of the LM and the TEM micrographs of this research are shown in figures one through fifteen. An explanation of them follows.

Figure 1 illustrated the leaf ultrastructure at the light microscope level. Micrographs a and d represent fresh tissue and they show three structural components; chlorenchyma cells, vascular bundle cells, and epidermal cells.

The chlorenchyma (Ch) cells are chloroplast containing cells. Within these cells are chloroplasts which appear as small gray

Figure 1. Light Micrographs of alfalfa leaf tissue.

a) fresh leaf tissue

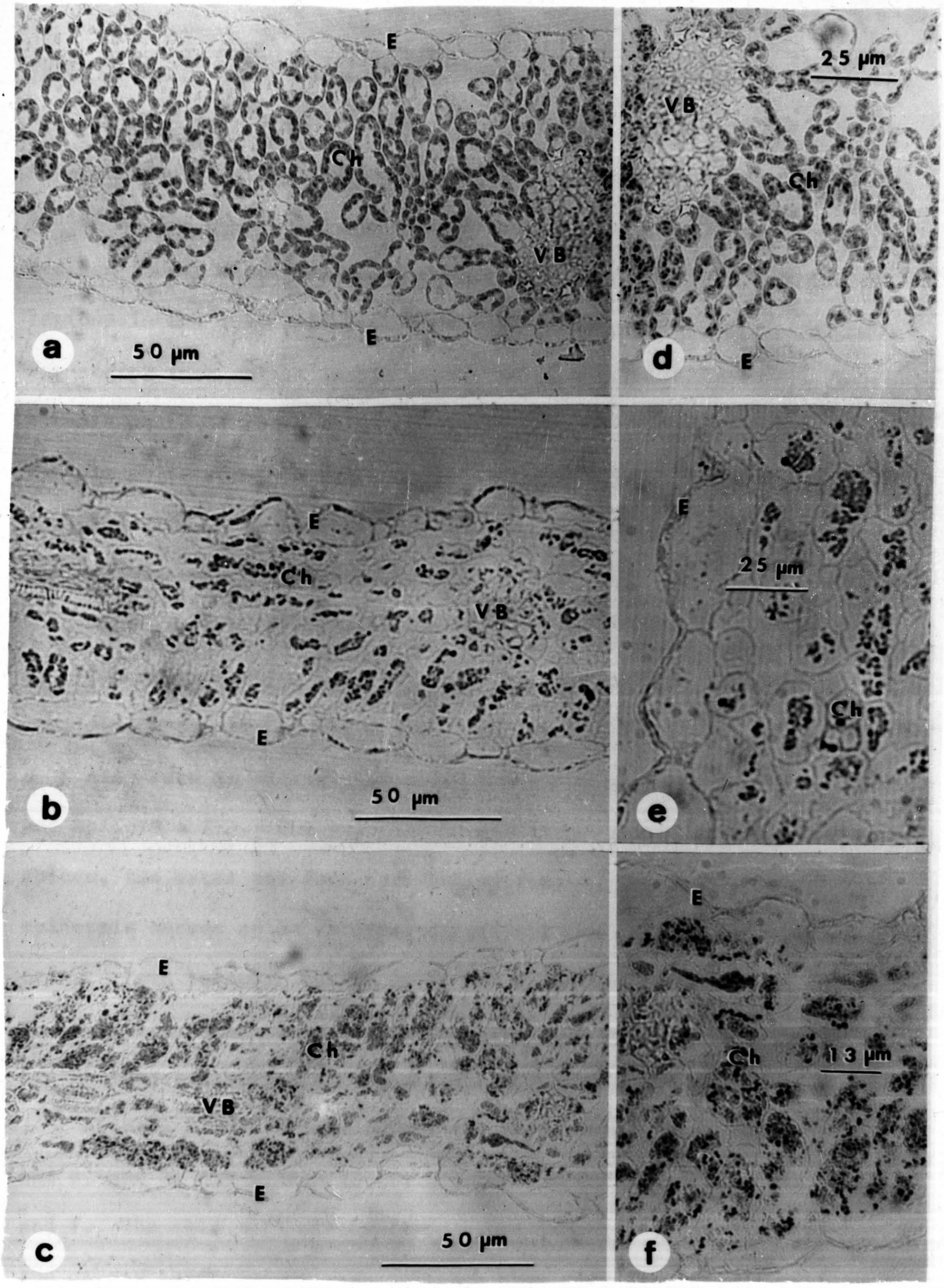
b) leaf tissue after 17 days ensiled

c) leaf tissue after 51 days ensiled

d) enlargement of micrograph a

e) enlargement of micrograph b

f) enlargement of micrograph c


E
Ch
VB
E
a
50 µm
25 µm
VB
Ch
d
E
E
Ch
VB
E
b
50 µm
E
25 µm
Ch
e
E
Ch
VB
E
c
50 µm
E
Ch
13 µm
f

spheres on the cell's periphery. Chloroplasts (C) are organelles which manufacture starch by photosynthesis and then store the starch as granules until it is needed. Since all LM was done using iodine staining, the starch granules present in the chloroplasts are stained darker than their surroundings and appear as small darkened regions in the grey spheres. Iodine is a differential stain specific for starch in a cell. In the center of each chlorenchyma cell is a vacuole which is bound by the tonoplast, a unit membrane. The vacuole is responsible for osmotic pressure control since it is involved with water uptake and maintenance of water in the cell. The tonoplast is not visible in this micrograph.

Micrograph a illustrates the large percent of the leaf occupied by chlorenchyma cells, and this accounts for the high value of available nutrients in the leaf.

Also seen in micrographs a and d are the vascular bundle (VB) and epidermis (E). The vascular bundle is comprised of xylem and phloem, the water and food carrying systems of the plant. The epidermis serves as an external structural and protective layer of cells. Both vascular bundle cells and epidermal cells appear empty in the micrograph. This is because the xylem and epidermal cells are no longer living after they reach maturity.

Aflalfa 17 day silage leaves are represented in micrographs b and e, and 51 day silage leaves are represented in micrographs c and f. The cell wall structure remained intact. These micrographs and others taken during this research show very little cell wall

decomposition during ensiling. This is attributed to the lack of celluloytic bacteria present in the silage microflora. In the chlorenchyma cells of b, c, e, and f, the cell ultrastructure is seen disrupted and decomposed. Note the lack of structure on the cell's periphery and the presence of cell material in the cell's center. However, even though the cell's ultrastructure is decomposed and disrupted, there are still darkened areas in the cell which represent starch. This is evident in micrographs e and f.

Breakdown in ultrastructure and cell walls may be due to enzymatic, methanical, or microbial. After the plant is cut it undergoes some enzymatic destruction. It is wilted after cutting and this causes cellular disruption by physical means. Finally, the cells are subjected to microbial decomposition both by a bacterial population outside the normal silo microflora before ensiling and by the silo microflora once ensiled. It is not possible to separate the breakdown, due to any one particular reason, when analyzing these micrographs. A more specialized study would be needed.

The alfalfa stem is shown in Figure 2. Micrographs a and d represent fresh stem. In micrograph a, the chlorenchyma (Ch) cells can be seen just inside the epidermal (E) and the collenchyma (Cl) cell layers. Both the epidermal and collenchyma cells serve as structural and protective cell layers covering the interior cells, and neither cell type is alive after maturity is reached. They also are composed of thicker cell walls than most cell types.

Figure 2. Light Micrographs of alfalfa stem tissue.

a) fresh stem tissue

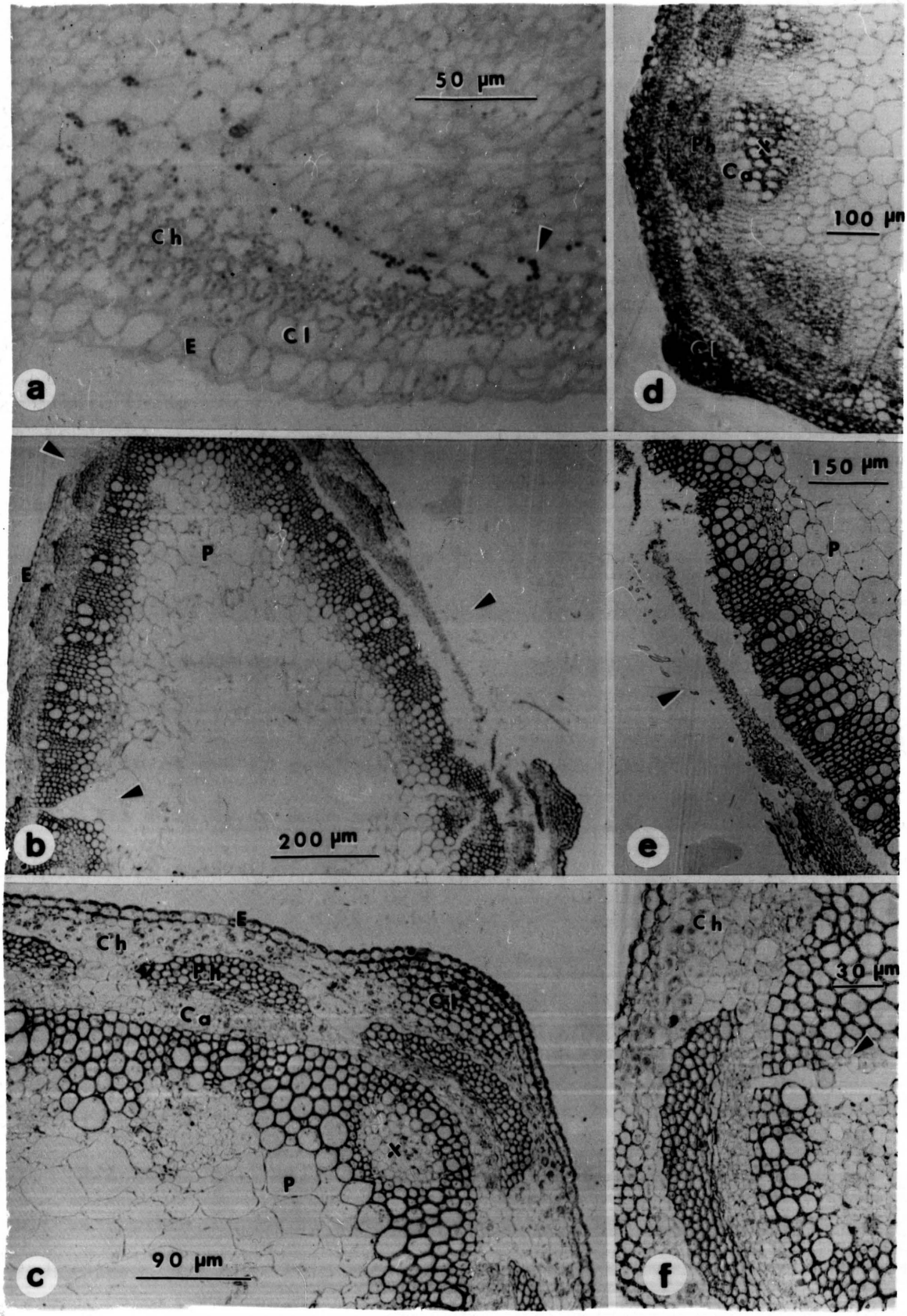
b) stem tissue after 17 days ensiled

c) stem tissue after 51 days ensiled

d) an overview of micrograph a

e) enlargement of micrograph b

f) enlargement of micrograph c


50 µm
Ch
E
Cl
a
d
P
Ca
100 µm
Cl
b
E
P
200 µm
e
150 µm
P
c
E
Ch
Ph
Ca
Cl
X
P
90 µm
f
Ch
30 µm

The chlorenchyma cells shown have the same structure as in leaf chlorenchyma. Starch granules appear as dark areas due to iodine staining and they are within chloroplasts which appear as grey areas on the cell's periphery. The arrow in micrograph a points to starch granules.

Micrograph d illustrates some of the other areas of the stem. The meristematic zone of a young stem is marked as collenchyma cells because these are the cells which comprise the growth point. Also pictured are the Xylem (X), Phloem (Ph), and Cambium (Ca). The cambium is the only growth area where new cells are continually developed instead of growth being due only to cell enlargement.

Seventeen day silage is represented by micrographs b and e. The arrows denote areas where cells are missing or separated. This can be due to either enzymatic, mechanical, or microbial causes but appears to be due to mechanical since the epidermis and collenchyma cells are missing in some parts but present in others. There is no evidence of erosion of cells.

The center of the stem is composed of parenthyma cells which make up the region called the pith (P). Sometimes these cells contain chloroplasts and are photosynthetic. During growth the center of the pith is often destroyed so cell wall breakage found in the pith is common at the stem's maturity.

All other cell walls in micrograph b appear intact, but cell ultrastructure cannot be seen clearly enough to determine its integrity.

In 51 day silage, represented by micrographs c and f, the structural integrity of the stem cell walls are seen clearly. All of the typical stem cell types are pictured, and the chlorenchyma cells shown contain disrupted cytoplasm. In micrograph f, however, the arrow indicates a break in the middle lamella of some of the xylem cells and cells surrounding the xylem. The middle lamella acts as a cell wall cement binding cell walls together.

The stem, after 17 days ensiled, is pictured again in Figure 3a. In this micrograph four meristematic (Ms) regions are shown. These regions are comprised of young collenchyma cells. Exterior to all the cells is a layer of epidermal cells. Just interior to the epidermis is a layer of collenchyma cells, but they are hard to recognize in this micrograph. The chlorenchyma cells occupy the next interior region after the epidermal and collenchyma cells. Then the phloem and xylem follow. These two vascular tissue cell types are separated by a region of cambium cells. The phloem and xylem appear as vascular bundles with chlorenchyma cells separating the bundles. During maturation the xylem cells will grow together, but the phloem and cambium will still be separated by chlorenchyma. The innermost cellular region of the stem is the pith, however, in this micrograph it is not clearly seen.

Micrograph c is an enlargement of a more mature stem, and it illustrates the pith region more clearly than micrograph a. The pith is composed of thin walled parenchyma cells, which when young are living, but as the stem matures they sometimes disrupt. The

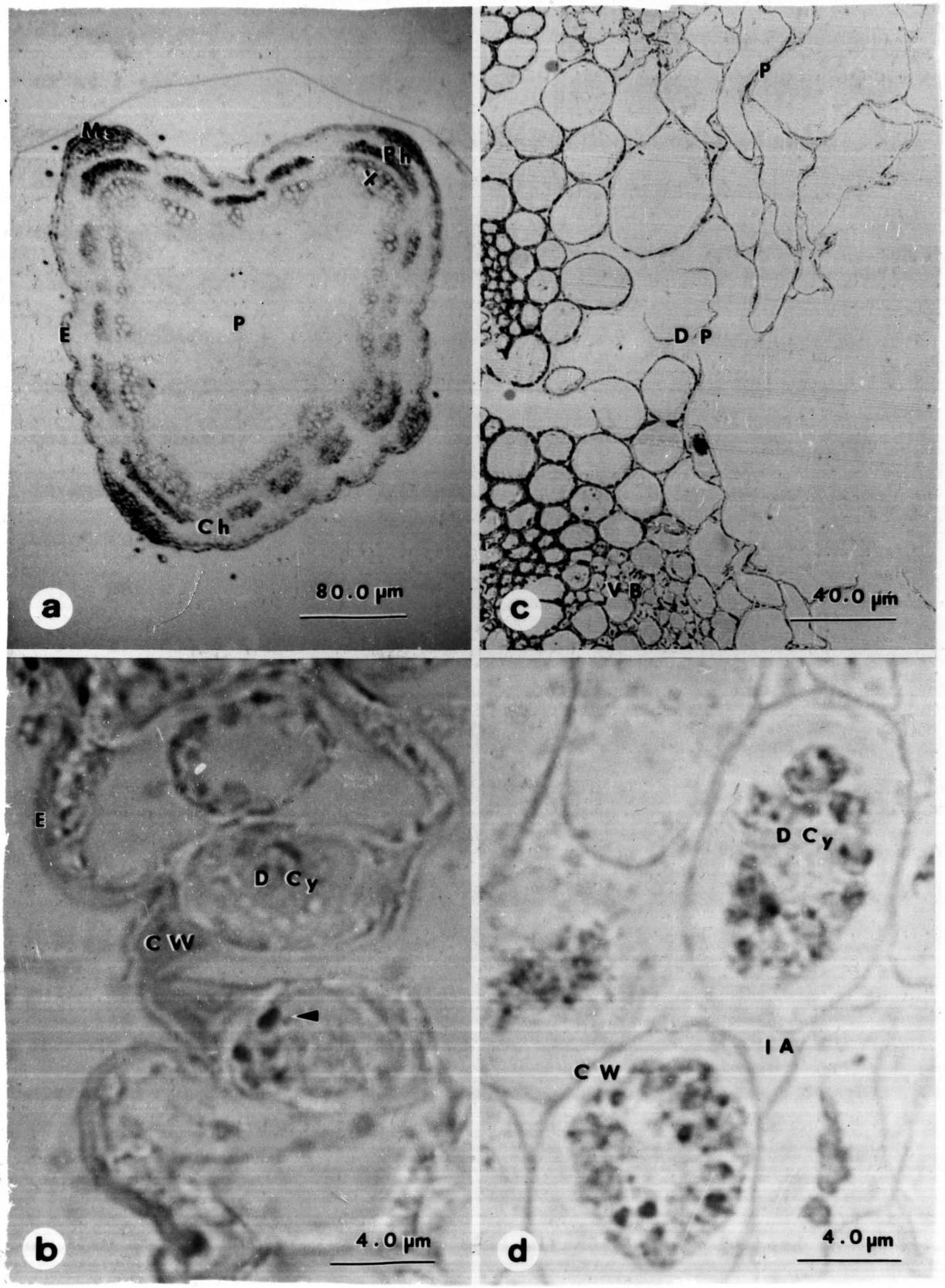
Figure 3. Light Micrographs of alfalfa stem tissue.

a) Cross section of stem tissue from 17 day silage

b) Cross section of a stomate from 17 day silage

c) Cross section of pith cells from 17 day silage

d) Cross section of chlorenchyma cells from 51 day silage


Ph
X
E
P
Ch
80.0 µm
a
P
D P
V B
40.0 µm
c
E
D Cy
CW
4.0 µm
b
D Cy
I A
CW
4.0 µm
d

disruption seen here may be due to a naturally occurring disruption or as a result of decomposition of cell walls during ensiling. However, this micrograph shows the intact pith cells void of ultrastructure which would indicate they were dead before ensiling and thus, the disruption would probably be due to natural disruption caused by maturation.

A stomate is illustrated in micrograph b. This is a pore in the epidermis where gas exchange can occur since these two guard cells are able to open and close allowing gas exchange. The arrow is located in a guard cell and is pointing at a darker stained area which is starch. On either side of the guard cells are subsidiary cells which help the guard cells to function. Guard cells and subsidiary cells are the only kind of cells in the epidermis which are alive and this is because of their function. Note the thick cell wall region of the guard cells and the presence of disrupted cytoplasm (DCy) in both the guard cells and the subsidiary on the sides of the guard cells. This micrograph is from a 17 day ensiled stem.

Micrograph d illustrates one of the highest magnifications possible with the light microscope. The cells shown are chlorenchyma cells of 51 day ensiled stem. Intracellular air space (IA) is present between cell walls of cells and within the cell walls is disrupted cytoplasm (DCy). Note the absence of the center vacuole and the lack of organelles. The cell wall, however, appears intact.

The light micrographs seen in Figures 1 to 3 show an overview of stem and leaf tissue, however, cell ultrastructure is hard to

identify. The transmission electron microscope allows the identification of ultrastructure since the lowest magnification generally shows only four to five cells at a time. This is similar in magnification to Figure 3d, which illustrates one of the highest magnifications of the light microscope.

Figures 4 through 15 illustrate fresh and ensiled alfalfa leaves and stems viewed by TEM. The magnifications used during TEM ranged from 3,000x to 35,000x. Much higher magnifications were possible but not necessary since enlargements were made of the negatives during the photographic procedures.

Figure 4 represents chlorenchyma cells from all of the six samples studied in this research. Micrographs a, b, and c represent fresh, 17 days ensiled, and 51 days ensiled alfalfa leaves, respectively. Similarily, micrographs d, e, and f represent fresh, 17 days ensiled, and 51 days ensiled alfalfa stems, respectively.

Micrographs a and d show the same ultrastructure which was visible in the light micrographs of Figure 1a and 1d, and Figure 2a. The cell wall encompasses the cell ultrastructure and within the cell walls are found chloroplasts, denoted by arrows, which contain starch granules (S). The vacuole (V) is located in the cell's center and cytoplasm encompasses the cell organelles which are located on the cell's periphery. Small strands of cytoplasm are seen passing through the vacuole of micrograph a. Separating the cells from one another is intracellular air space (IA), and joining the cell walls of one cell to another is middle lamella.

Figure 4. Transmission Electron Micrograph cross sections of typical Chlorenchyma cells from alfalfa leaves and stems.

a) A chlorenchyma cell from a fresh leaf

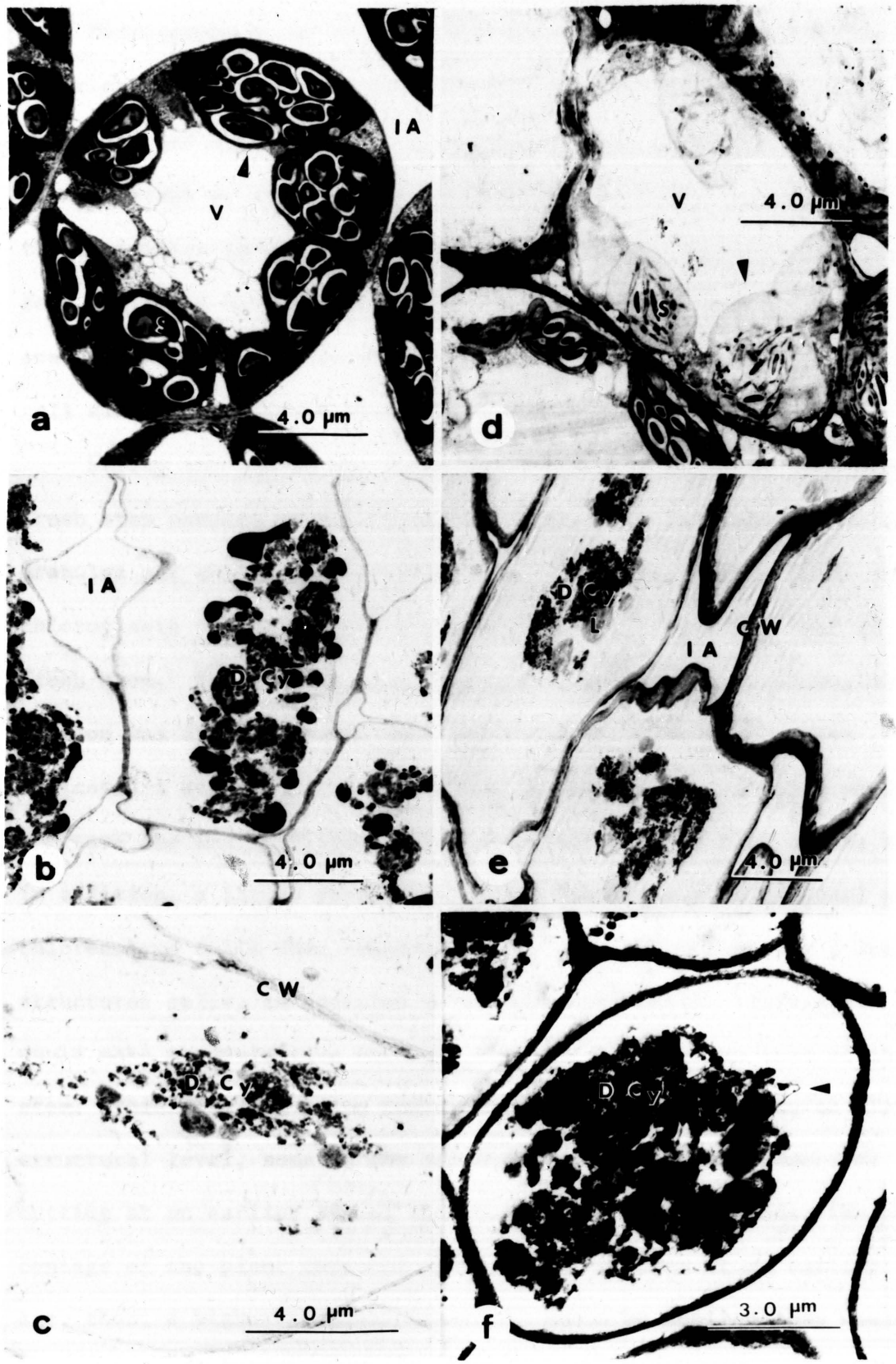
b) A chlorenchyma cell from a 17 day ensiled leaf

c) A chlorenchyma cell from a 51 day ensiled leaf

d) A chlorenchyma cell from a fresh stem

e) A chlorenchyma cell from a 17 day ensiled stem

f) A chlorenchyma cell from a 51 day ensiled stem


IA
V
a
4.0 µm
V
4.0 µm
d
IA
D Cyl
b
4.0 µm
D Cyl
CW
IA
e
4.0 µm
CW
D Cyl
c
4.0 µm
D Cyl
f
3.0 µm

Micrographs b, c, e, and f all show similar chlorenchyma cells after ensiling. The cell walls remain intact, but the cytoplasm is disrupted and a vacuole no longer occupies the center of the cell. In micrograph e, lipid (L) is present. This is the one component of the cell which is not readily apparent in the living cell, but is seen easily in disrupted cytoplasm. This fact is attributed to a coal escence of lipid after death due to a hydrophobic effect in the cell since lipid is not generally decomposed by bacteria.

During the use of the TEM a count was made of fresh leaf and fresh stem samples of the chloroplasts per cell section and the starch granules per chloroplast section (Table 4). The average count for chloroplasts per cell section was nine in fresh leaf and four in fresh stem. The average count for starch granules per chloroplast section was eight in fresh leaf and three in fresh stem. This indicates a higher starch production in the leaf cells which would increase the leaf nutritive value considerable over that of the stem. In addition, a larger percentage of the leaf tissue is composed of chlorenchyma cells when compared to the stem which contains a lot of structural cells, in addition to chlorenchyma cells. This again would make the nutritive value of the leaf higher than that of the stem. This data are quite important since it supports, on an ultra-structural level, some of the research on silage which advocates cutting at an earlier age of the plant for ensiling because the percentage of the plant composed of leaves is greater at an earlier age, thus, a higher nutrient feedstuff would be ensiled than would

TABLE 2. Starch and chloroplast counts.[a]

Tissue	Starch granules per chloroplast section		Chloroplasts per cell section	
	leaf	stem	leaf	stem
Fresh alfalfa	8	3	9	4
17 day ensilage	Some starch granules seen, but not within intact chloroplasts		undefinable due to decomposition of intracellular components	
51 day ensilage				

[a]Counted while viewing sections under the TEM.

be at a more mature stage.

Within both the leaf and stem there are many cell types and organelles. Figure 5 illustrates some of these cells and organelles. In micrograph a, a nucleus (Nu) is the major organelle seen. A mitochondrion (M) is also present. They are bound on one side by cell wall and on the other sides by vacuoles.

The chloroplast (C) shown in micrograph d is active as indicated by the starch (S) present which was produced on the granum (G) during photosynthesis. This chloroplast lies next to a cell wall (CW) on one side and a membrane (Me) on the other side. This membrane is called the tonoplast. It is a specialized membrane which encompasses the vacuole. To relate this to previous micrographs, in Figure 4 the living cells in micrographs a and d contain organelles on the inside periphery of the cell wall and the cell's center is occupied by a vacuole. The tonoplast functions in osmotic regulation of the vacuole. This integrity was lost during ensiling and micrographs b, c, e, and f of Figure 4 illustrate the disruption of the cytoplasm and organelles.

Micrographs b and c of Figure 5 illustrate a transfer cell (TC). The transfer cell is a specialized cell which is designed to allow more nutrients to enter the cell. This is illustrated most clearly in micrograph c and Figure 6, which are enlargements of one of the cell walls seen in micrograph b. Labyrinth (La) are protrusions from the cell wall which increase membrane surface area thus, allowing for increased transfer ability. The transfer cell is believed

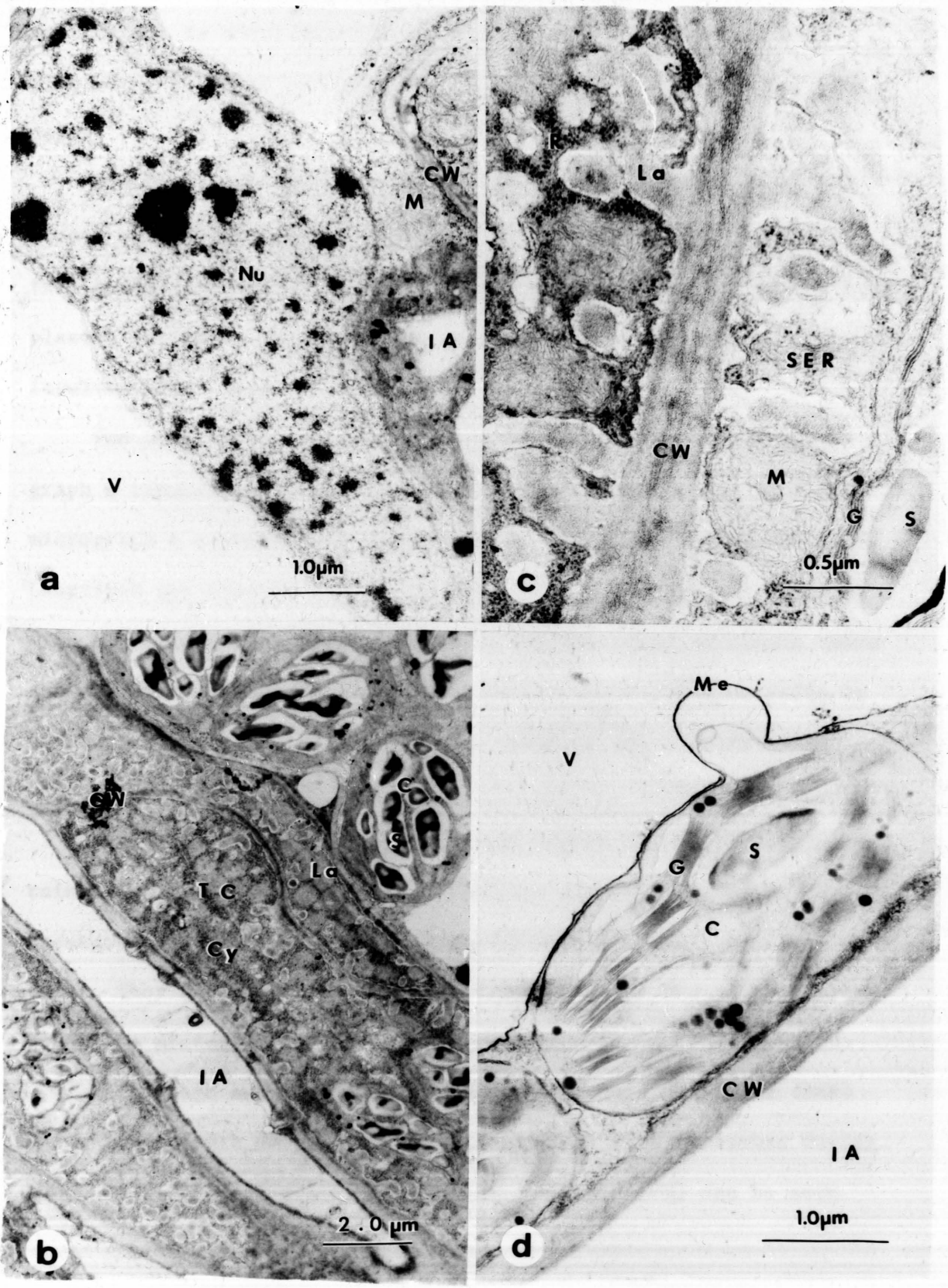
Figure 5. Transmission Electron Micrographs of various types of cells and cell ultrastructure.

a) Cross section of a nucleus from fresh leaf

b) Longititudinal section of a transfer cell from fresh leaf

c) Cross section of a cell wall from fresh leaf

d) Cross section of a chloroplast from fresh stem


a
Nu
CW
M
IA
V
1.0µm
c
La
SER
CW
M
G
S
0.5µm
b
CW
C
La
TC
Cy
IA
2.0µm
d
M-e
V
G
S
C
CW
IA
1.0µm

to function in short distance solute transferring and they are frequently found as parenchyma cells bordering the phloem and xylem cells.

Figure 6 also shows some other organelles; ribosomes (R) and smooth endoplasmic reticulum (SER). Ribosomes function during protein synthesis and are composed of protein and RNA. The smooth endoplasmic reticulum is a membrane system within the cytoplasm and is involved with ribosomes during protein synthesis.

Two more transfer cells are represented in Figure 7. Micrograph a represents a transfer cell from a 17 day ensiled leaf and micrograph b represents a transfer cell from a 51 day ensiled leaf. Labyrinth are clearly visible and appear to be intact and continuous with the cell wall in most cases. In the disrupted cytoplasm there are starch granules and grana still intact. The transfer cells in micrograph b represent either phloem cells or xylem cells which have not yet matured and died.

More organelles are illustrated in Figure 8. Micrograph a contains a microbody (Mb). These organelles either function in sugar breakdown or lipid breakdown, depending upon which enzymes they contain. They are generally found near chloroplasts and in this micrograph the microbody is between a chloroplast and a lipid droplet.

Micrograph b contains some rough endoplasmic reticulum (RER) which like smooth endoplasmic reticulum works with ribosomes during the formation of proteins. The plasma membrane (PM) can be seen

Figure 6. Transmission Electron Micrograph of a transfer cell from fresh leaf.

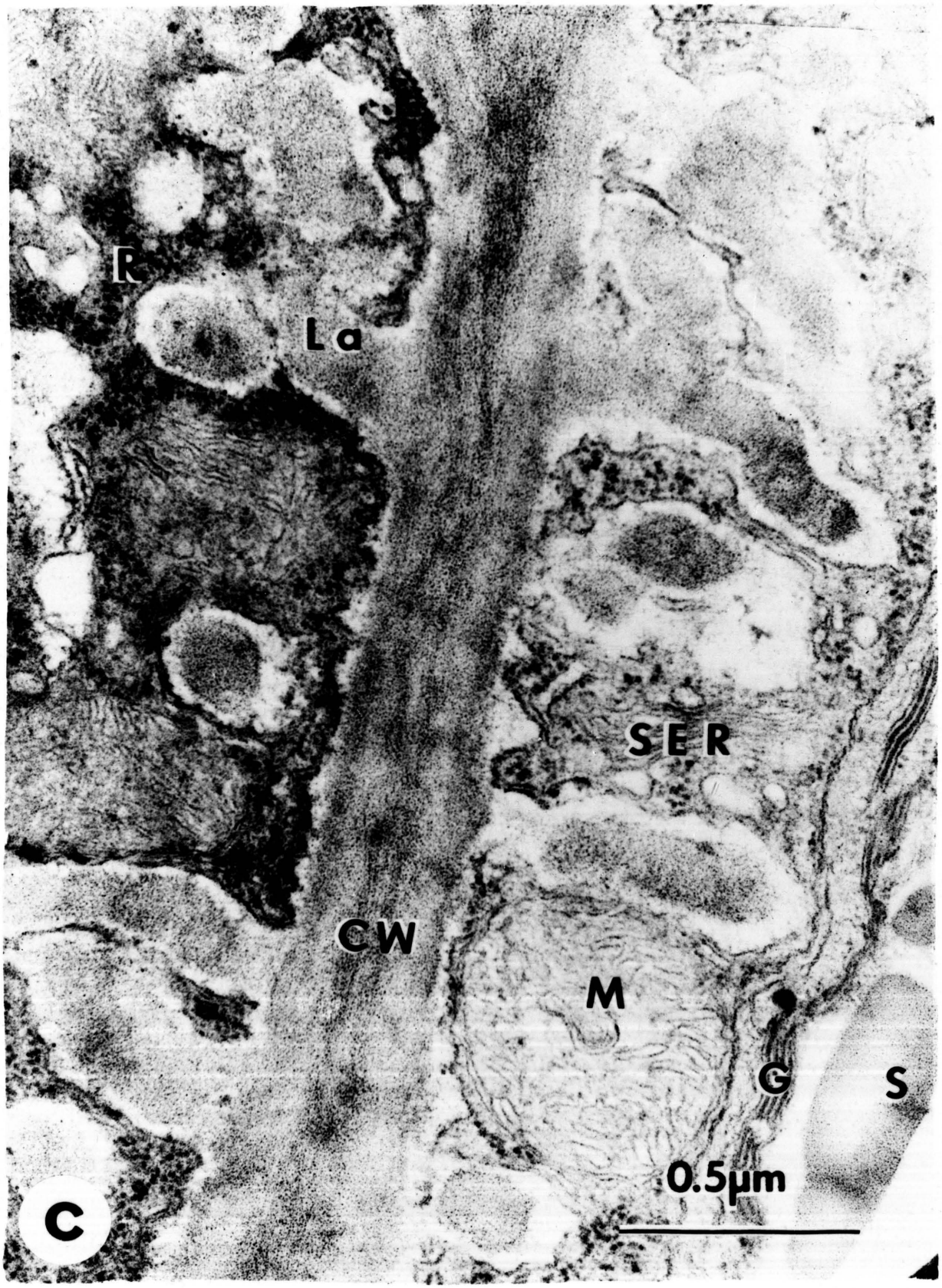

R
La
SER
CW
M
G
S
0.5µm
C

Figure 7. Transmission Electron Micrographs of transfer cells from leaf 17 and 51 day silage.

a) Cross section from a 17 day ensiled leaf

b) Cross section from a 51 day ensiled leaf

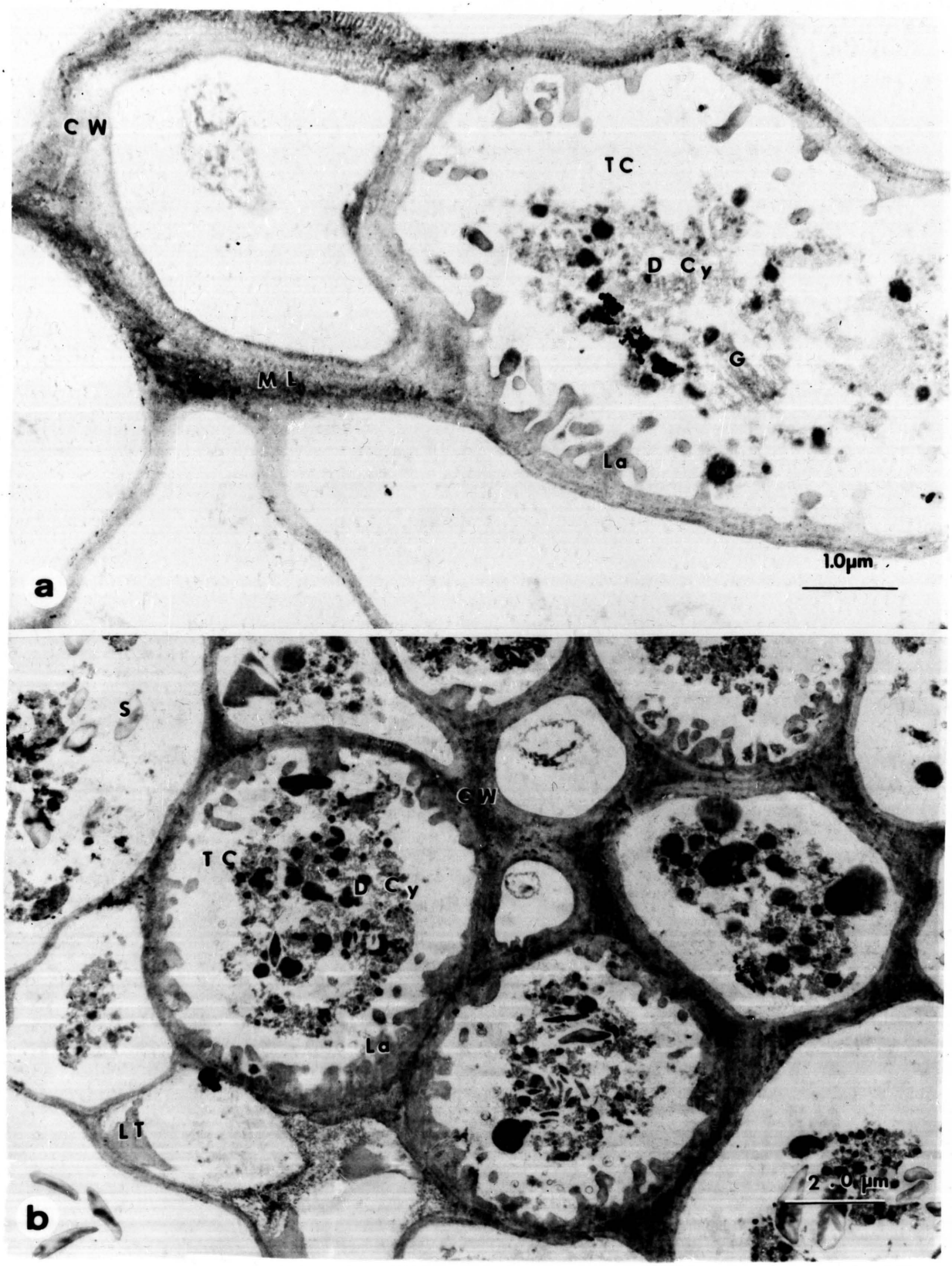
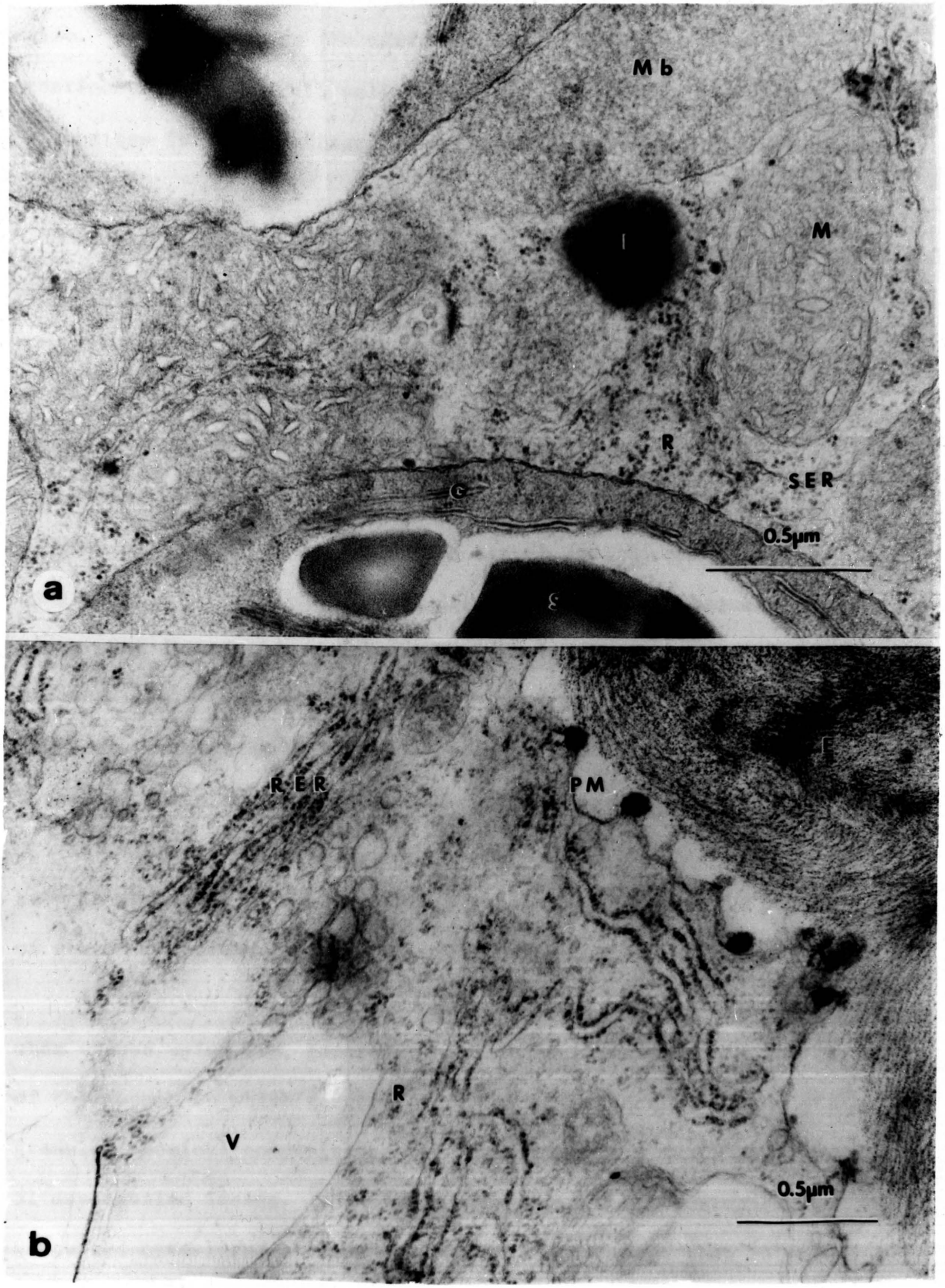

C W
TC
D Cy
G
M L
La
1.0µm
a
S
C W
TC
D Cy
La
LT
2.0µm
b

Figure 8. Transmission Electron Micrographs of leaf and stem cell ultrastructure.

a) Cross section from fresh leaf

b) Cross section from fresh stem


Mb
M
R
SER
0.5µm
a
RER
PM
R
V
0.5µm
b

also. It is joined to the cell wall in certain points and is not continuous with the cell wall between the two points. Both micrographs are from fresh tissue, a representing leaf and b representing stem.

Chloroplasts from each tissue type are represented in Figure 9. Micrographs a, b, and c are leaf tissue; a representing fresh, b representing 17 days ensiled, and c representing 51 days ensiled leaf. Micrographs d, e, and f are stem tissue and they represent fresh, 17 days ensiled, and 51 days ensiled, respectively.

Chloroplasts are mostly found in chlorenchyma cells and are the organelles capable of manufacturing starch (S), by photosynthesis, on granum (G). Mitochondria (M) are generally seen near chloroplast since they function in respiration which involves the catabolism and anbolism of metabolic products. In micrograph d, a mitochondrion has been invaginated by the chloroplasts, and another is seen at the bottom of the micrograph. Cytoplasm surrounds the chloroplasts.

In micrographs b, c, e, and f traces of grana (G or arrow) are seen in the cellular debris. Micrograph c illustrates the presence of starch in some cells even after 51 days ensiled.

An enlargement of starch is seen in Figure 10. Starch granules when viewed using TEM, generally have cross lines which is indicative of the manner in which electrons are reflected because of the starch granules chemical composition. Both micrographs a and b are from 51 day ensiled leaves. The starch granules are all surrounded by disrupted cytoplasm. It is hard to explain the fact that intracellular

Figure 9. Transmission Electron Micrographs of Chloroplasts from alfalfa leaves and stems.

a) Cross section of a chloroplast from a fresh leaf

b) Cross section of a chloroplast from a 17 day ensiled leaf

c) Cross section of a chloroplast from a 51 day ensiled leaf

d) Cross section of a chloroplast from a fresh stem

e) Cross section of a chloroplast from a 17 day ensiled stem

f) Cross section of a chloroplast from a 51 day ensiled stem

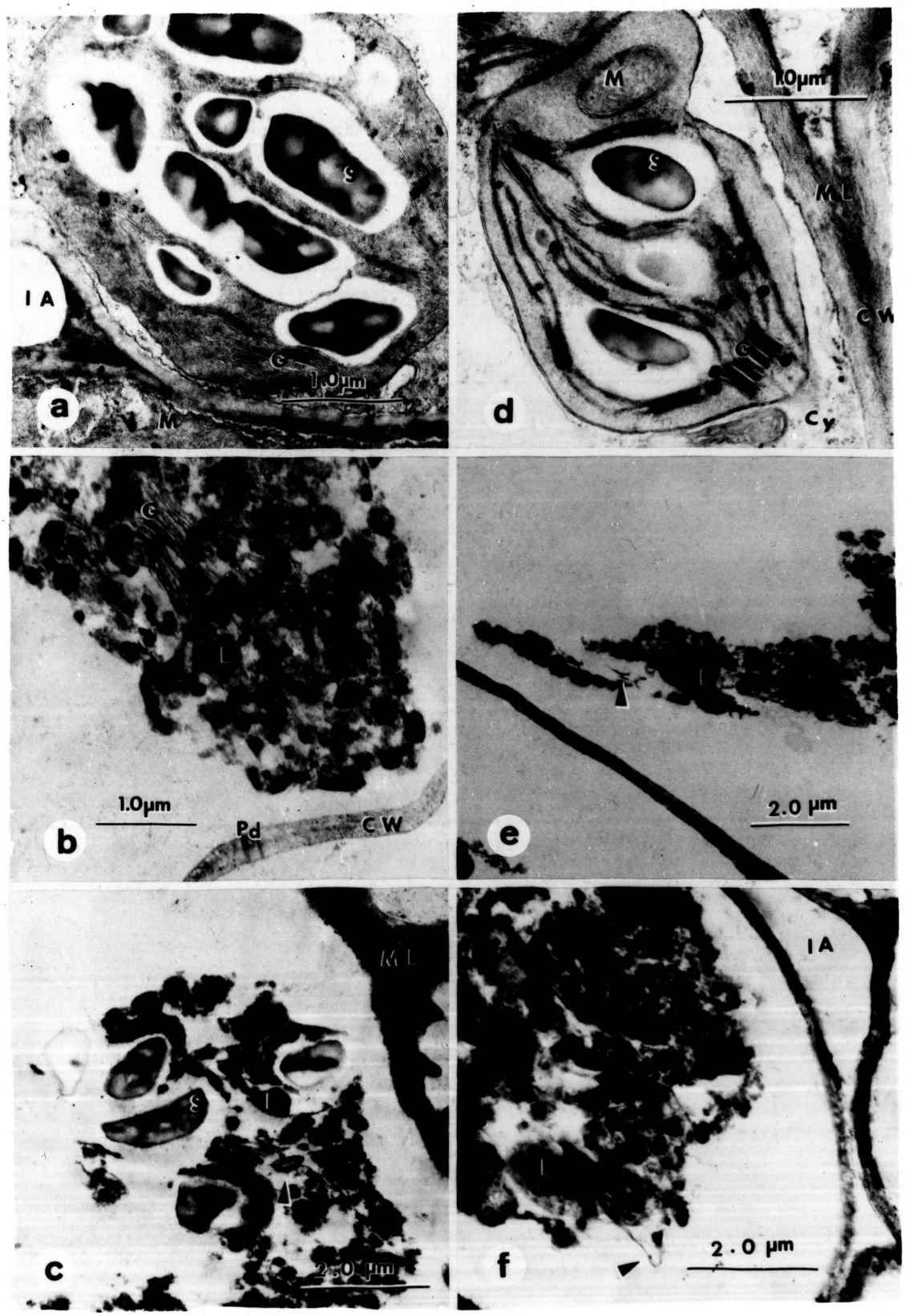
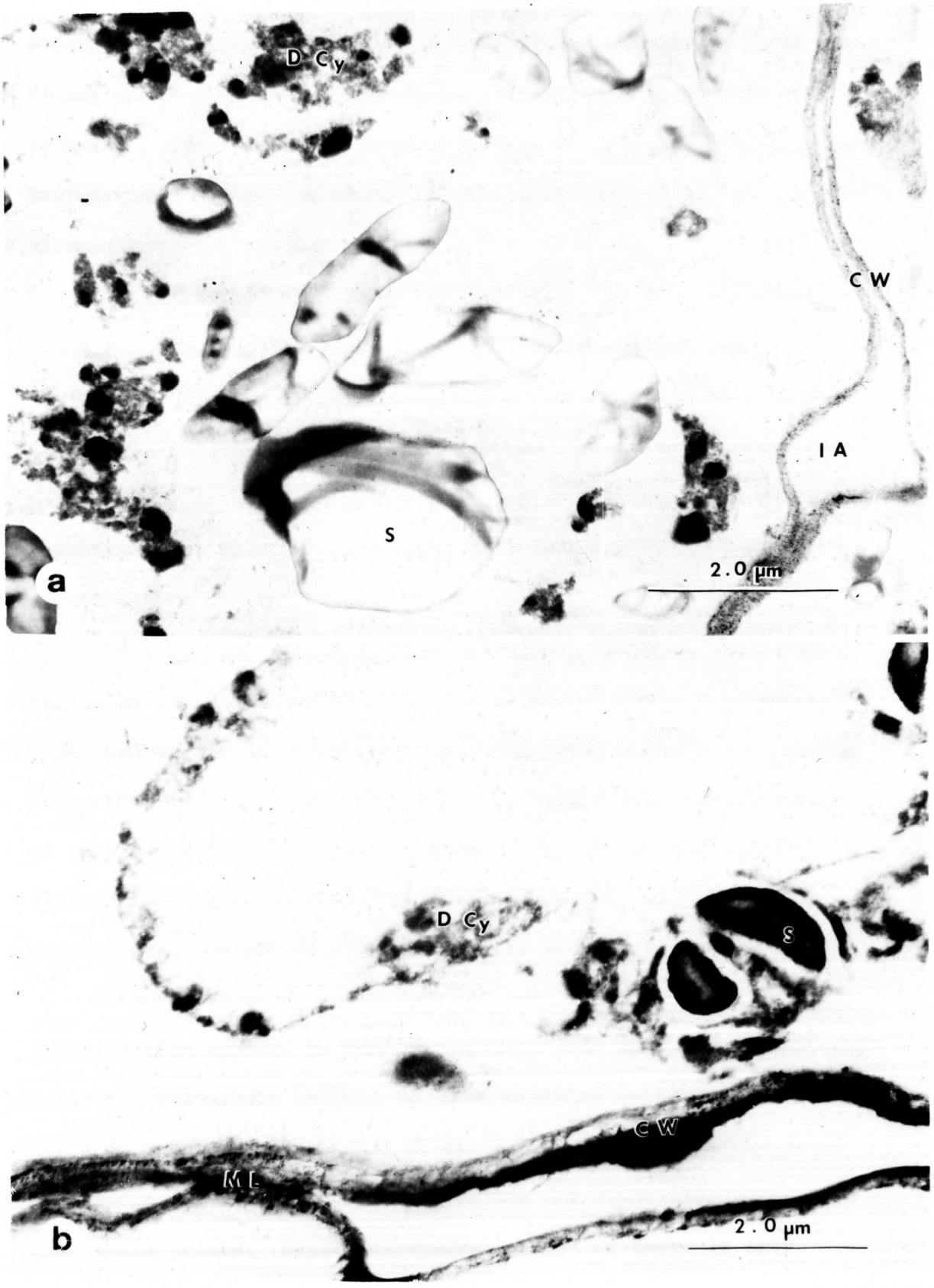

a
IA
S
C
1.0μm
M
d
M
1.0μm
S
ML
CW
C
Cy
b
C
L
1.0μm
Pd
CW
e
L
2.0 μm
c
ML
S
L
2.0 μm
f
IA
L
2.0 μm

Figure 10. Transmission Electron Micrographs of Starch Granules found in 51 day leaf silage.


D Cy
CW
IA
S
2.0 µm
a
D Cy
S
CW
ML
2.0 µm
b

structures are decomposed yet starch granules are intact. One theory is that the decomposition of the cell ultrastructure is enzymatic where starch still remains because it seems unlikely that starch would remain undigested after a microbial attack of the cell's ultrastructure.

The cell wall, as illustrated in Figure 11, is a structure which has remained intact during the ensiling process except in a few cases. Micrographs a, b, and c are leaf cell walls; a representing fresh, b representing 17 days ensiled, and c representing 51 days ensiled leaf. Micrographs d, e, and f are stem cell walls and they represent fresh, 17 day ensiled, and 51 day ensiled stem, respectively.

The cell wall is composed of two layers, primary and secondary, with the secondary layer on the interior of the primary layer with respect to cell contents. In micrographs a and b two primary cell walls are cemented together by the middle lamella (ML) which is composed primarily of pectic substances. It is not possible to differentiate between primary and secondary cell walls in these micrographs. On either side of the cell walls are cell contents.

Micrographs b and e show the cell wall as intact, but some decomposition appears to have occurred in cell walls of micrographs c and f. The middle lamella is quite distinct in micrograph e since there is a natural separation of the cell wall. The thickness of the middle lamella increases just before the separation of the two cell walls occurs. Unlike micrographs a and d, there is only

Figure 11. Transmission Electron Micrographs of Cell Walls from alfalfa leaves and stems.

a) Cross section of a cell wall from a fresh leaf

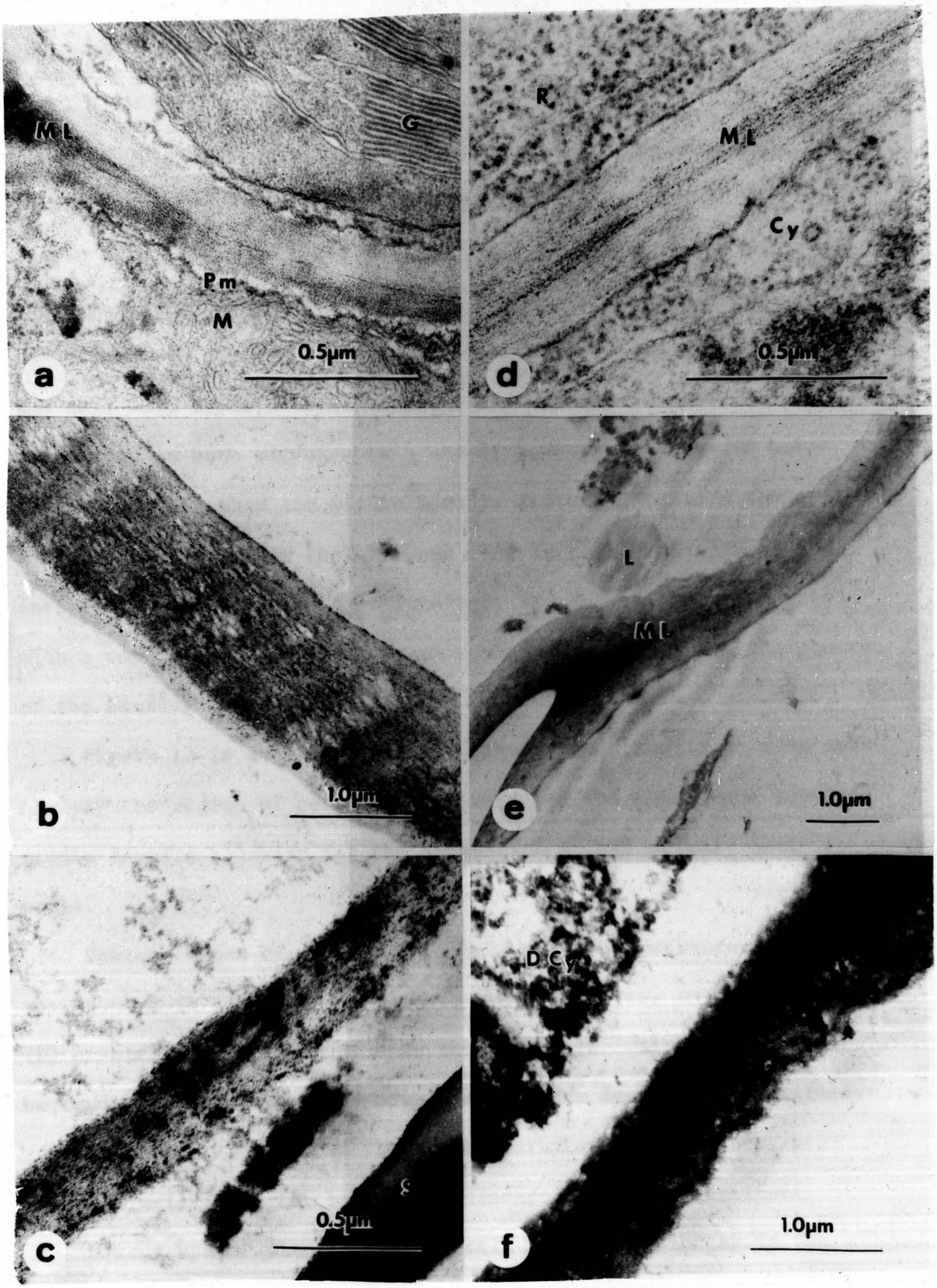
b) Cross section of a cell wall from a 17 day ensiled leaf

c) Cross section of a cell wall from a 51 day ensiled leaf

d) Cross section of a cell wall from a fresh stem

e) Cross section of a cell wall from a 17 day ensiled stem

f) Cross section of a cell wall from a 51 day ensiled stem


M L
G
Pm
M
0.5µm
a
R
M L
Cy
0.5µm
d
1.0µm
b
L
M L
1.0µm
e
0.5µm
c
D Cy
1.0µm
f

disrupted cytoplasm on either side of the cell wall of micrographs b, c, e, and f.

Although most cell walls were seen intact, there was some cell wall breakage apparent which is illustrated in Figure 12. Micrographs a, b, and c represents 17 day ensiled leaves and d, e, and f represent 17 day ensiled stems. The arrows in micrographs a, e, and f point to areas which appear to the eroded. In micrograph d, the cell wall appears to be both cut and eroded. One of the two cell walls in both micrographs b and c, however, appears tc have been broken and then the middle lamella destroyed between the cell walls. The reason for the breakage seen in these micrographs is unexplainable by this study's findings, and only a study conducted with a more controlled environment could possibly explain the causes of the breakage.

Figure 13 is an enlargement of Figure 12c. It illustrates more clearly the region of cell wall breakage and the subsequent loss of middle lamella allowing for the separation of the primary cell walls.

Various types of ensiled cell wall and other structures are represented in Figure 14. Micrograph a illustrates an unidentified (U) crystalline arrangement near the cell wall. Although it can't be truly defined, there are some speculations for the unidentified cell contents. They could represent disrupted chloroplasts, proteins or cellulose; the bacteria actinomycites; or an artifact.

Micrograph b illustrates pith cell walls and a break in the

Figure 12. Transmission Electron Micrographs of Cell Wall Breakage in 17 day ensiled leaf and stem.

a) Represents 17 days ensiled leaf

b) Represents 17 days ensiled leaf

c) Represents 17 days ensiled leaf

d) Represents 17 days ensiled stem

e) Represents 17 days ensiled stem

f) Represents 17 days ensiled stem

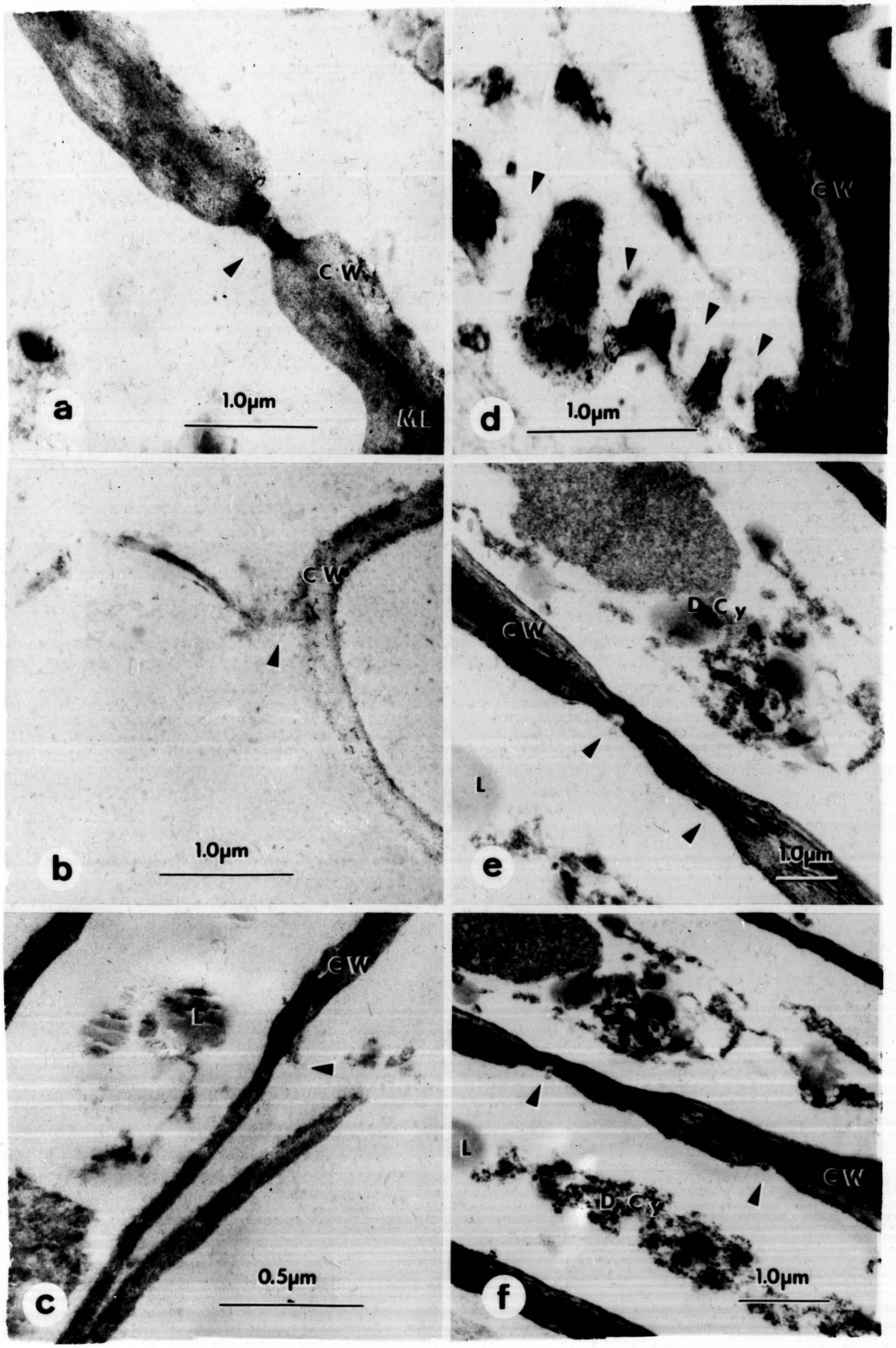

a
CW
ML
1.0μm
d
CW
1.0μm
b
CW
1.0μm
e
CW
D Cy
L
1.0μm
c
CW
L
0.5μm
f
L
CW
D Cy
1.0μm

Figure 13. Transmission Electron Micrograph of Cell Wall Breakage in 17 day ensiled leaf.

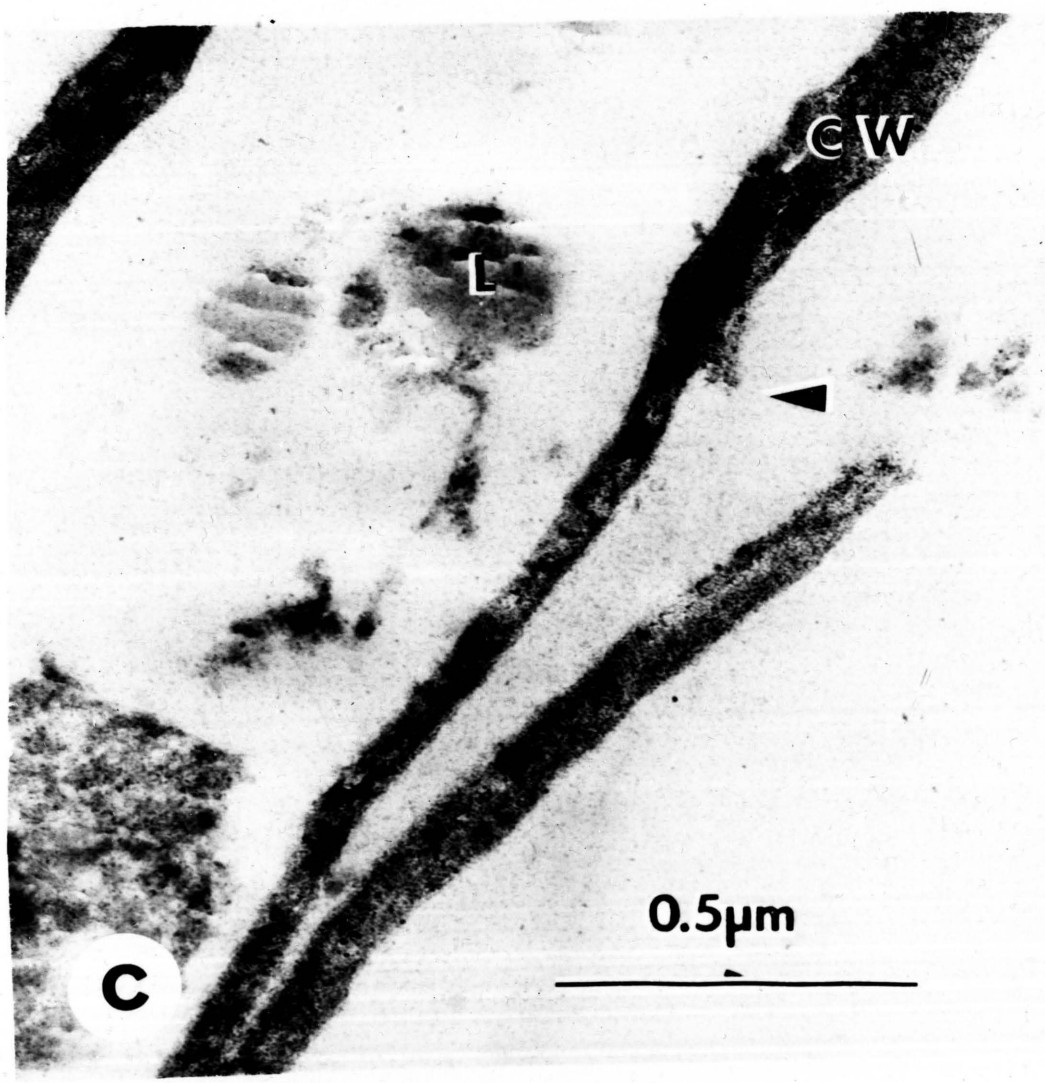

CW
L
0.5µm
C

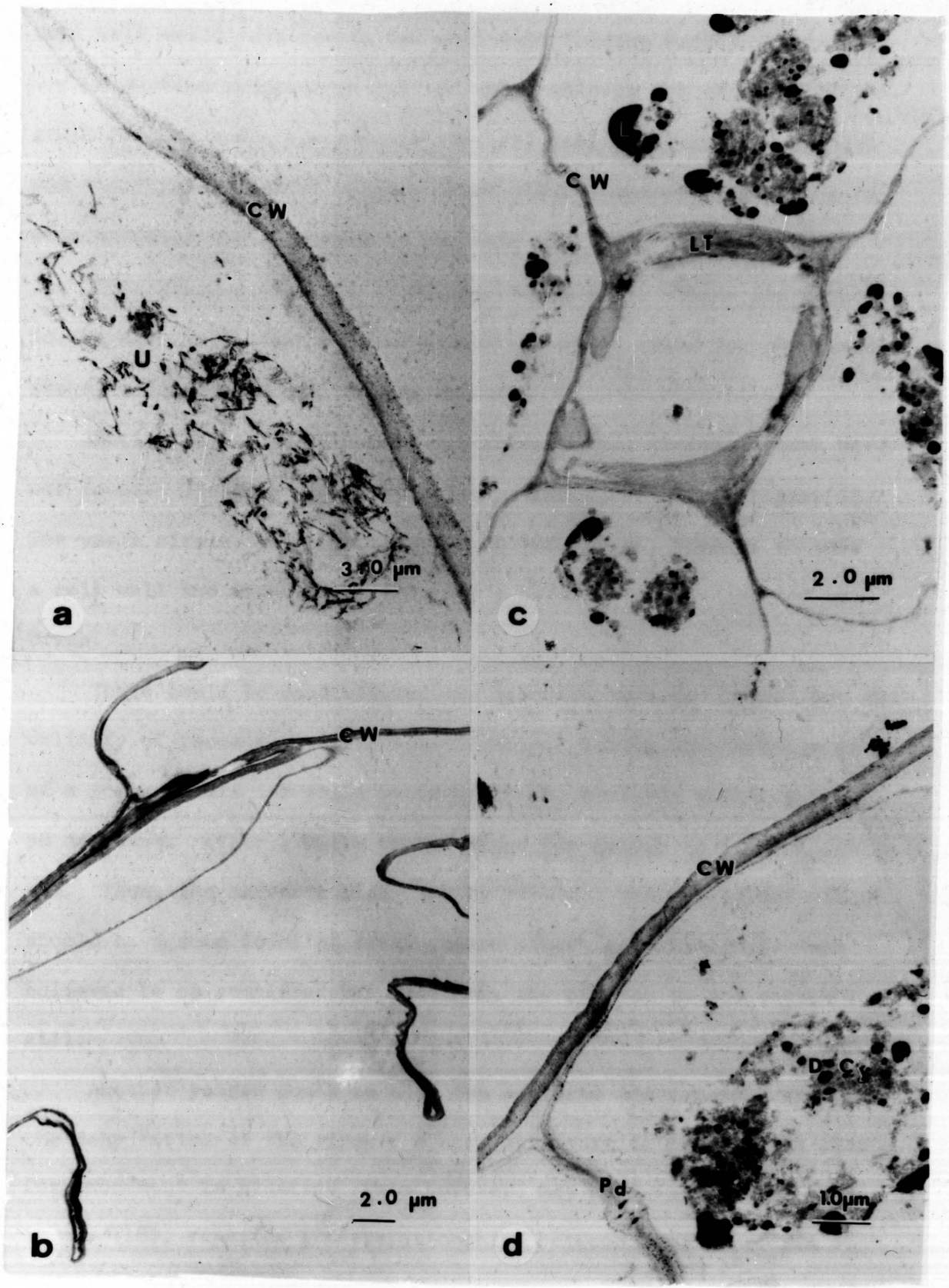
Figure 14. Transmission Electron Micrographs of Cell Walls From Different Tissue Regions.

a) represents 17 day ensiled leaf

b) represents 17 day ensiled stem

c) represents 17 day ensiled leaf

d) represents 17 day ensiled leaf


CW
U
3.0 µm
a
CW
LT
2.0 µm
c
CW
2.0 µm
b
CW
D-Cy
Pd
1.0µm
d

cell wall which resulted in the cell wall folding back on itself.

Lignified thickenings (LT) of xylem vessels are shown in micrograph c. These are areas where the cell wall had increased in size and its lignin content. They are not always connected from side to side but they are connected to the cell wall.

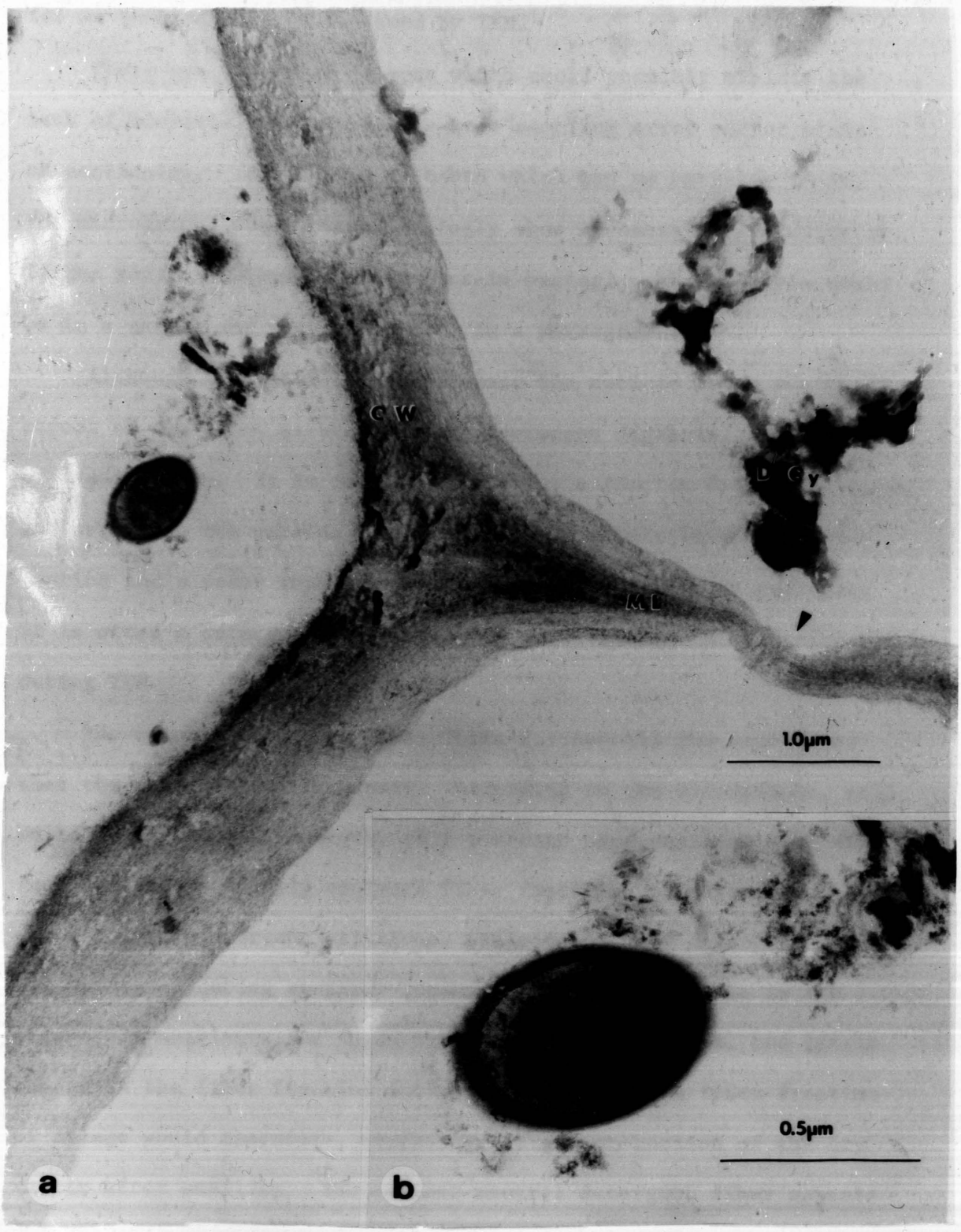
Micrograph d illustrates plasmodesmata (Pd). These are pores in the cell wall lined by plasma membrane which allow for the transfer of solutes from one cell to another.

During the LM and TEM work for this thesis, microorganisms were not found. The only possible microorganism was found in Figure 15. The small circle, which is enlarged in the insert, appears to have a cell wall and membrane or possibly a slime or spore coat and membrane.

There could be many reasons why bacteria were not found, but the validity of these reasons is questionable. During the fermentation of a forage there are acids produced by the bacteria which cause a pH decrease. After a while the pH drops low enough to kill bacteria. Thus, the bacteria might not be present, yet the 17 day silage should have some level of fermentation since fermentation is not believed to be completed yet and thus, the pH drop is not enough to kill.

Another reason could be that the bacteria are washed away during the dehydration of the tissue; a step necessary to prepare the tissue for embedment in plastic. This however, may be invalid since Akin et al. (10), reported interrelationships between rumen bacteria

Figure 15. Transmission Electron Micrograph of a 51 day Silage Cell Wall and a Probable Microorganism.


CW
DCy
ML
1.0µm
0.5µm
a
b

and cell walls after the cell walls had been exposed to rumen fluid for as long as 72 h, and viewed by TEM.

There are two other reasons which could possibly explain the lack of bacteria. These reasons are: sampling error and/or plane of sectioning. If a sample is taken which has no bacteria in it, the micrographs would correspondingly show no bacteria. Similarily, if the sections viewed did not contain bacteria, the bacteria could be in a sample and yet not be seen in a micrograph.

Although not previously mentioned, the cuticle is an important aspect of the plant structure which decreases digestibility because of its function. It is composed of cutin, a complex fatty substance, and surrounds the outside surface of the epidermis serving as protection and a water repellent. Bacteria can not degrade cutin and it is often a deterent to decomposition. It was not distinguishable during TEM.

The compositional analyses, Table 2, supports the same ideas that the micrographs illustrate. According to the micrographs, cell walls appear intact, however, cell contents upon ensiling were disrupted. The chemically analyzed fiber fractions support this. The percent lignin, percent cellulose, and percent acid detergent fiber all increased during ensiling because there is a decrease in the digestible nutrients due to decomposition by microflora, but little change in the fiber fraction during ensiling, so the fiber fraction of silage would therefore, comprise a larger proportion of the dry matter after ensiling. The percent neutral detergent fiber appears

TABLE 3. Compositional analyses (all values on dry matter basis).[11]

Sample	% Acid detergent fiber	% Lignin	% Cellu-lose	% Insoluble ash	% Neutral detergent fiber	% Ether extract	Dry matter	% Protein
Fresh alfalfa	26.10	5.64	19.24	1.22	39.55	1.55	19.5	24.86
17 day ensilage	30.95	9.05	21.48	.33	39.02	2.16	32.3	22.01
51 day ensilage	35.07	9.77	24.73	.56	39.59	2.50	32.3	20.87

stable because unlike the other fiber fractions, it is partially digested so its precent did not increase but by coincidence remained the same after ensiling. Very little ether extract was digested so after ensiling it represented a larger percent of the dry matter than it did before ensiling. There was an increase in dry matter after ensiling because the fresh tissue was wilted to decrease the percent of fluids in the tissue. The percent protein decreases during ensiling due to decomposition by the microflora. The decrease in insoluble ash is probably due to losses which occur during seepage.

The starch analysis (Table 3) of the samples further support the above conclusion. Fresh alfalfa contained more starch than 17 day silage, which contained more starch than 51 day silage. This indicates a breakdown in starch during ensiling and this breakdown is attributed to the decomposition caused by the silo microorganisms.

The research of this thesis shows many areas which need further research in order for the questions to be answered. The causes of breakdown in cell structure could be determined with more restricted research. An example would be to study changes occurring after cutting, after wilting, after 1 day ensiled, and after several days ensiled. It might then be possible to more precisely determine what forces caused certain decomposition which was noted in this research.

The use of TEM and LM has demonstrated the ability of these instruments to be used as tools if they are also supported by a chemical analysis. Scanning electron microscopy (SEM) could also

TABLE 4. Starch values.[23]

Tissue	Glucose concentration (µg/ml)
Fresh alfalfa	89.20
17 day ensilage	47.12
51 day ensilage	34.45

be used in studies such as these as an overview tool. The SEM scans surfaces and could give evidence of areas and methods of decomposition occurring during ensiling.

Current silage research is exploring chemical additives to silage as a means of increasing silage digestibility. Light microscopy, TEM, SEM, and chemical analysis could be used as tools to determine the chemical changes in the cell ultrastructure and it may be possible to alter the composition of the added chemicals to increase the digestibility of silage.

Silage research in previous years has made insufficient changes to significantly improve our present ensiling techniques or to enlighten us more on the ultrastructural methodology of the ensiling process. Continued silage research is important, however, because silage is a means of preserving forage in a manner to yield more dry matter per unit than hay and is also cheaper to harvest and store. Microscopic analysis can increase the understanding of ensiling and consequently, may lead to improved ensiling methods resulting in an increase of nutrients available to the ruminant from silage.

This study has visually shown changes which have occurred after the ensiling of alfalfa. Visual observation was important because it emphasized changes which occurred and showed where future research concerns should be in order to better understand the ultrastructural changes occurring during ensiling.

Even though this research was exploratory, the results showed

that microscopic analysis agreed with chemical analysis, and further showed that given a more controlled experiment more data could be gathered from micrographs. The three factors affecting integrity were: mechanical, microbial, and/or enzymatic. Changes caused by these three factors could be resolved under more specific conditions, and that would lead to ways the initial forage could be treated to enhance the resulting ensiling process. This research showed the practicality of looking at silage and future studies should stem from this research.

SUMMARY

The purpose of this study was to observe structural changes occurring during ensiling using LM and TEM, and then to evaluate the use of the LM and TEM as research tools. From the results of this research it can be concluded that the light and transmission electron microscope analysis agreed with the chemical analysis. The use of the microscopes, however, allowed a different means of observation, that means being visual. Chemical analysis quantitatively expressed the changes which occurred, but it did not determine the methods by which the changes occurred. The microscope presented a view of the actual changes which had occurred during ensiling and could allow an understanding of the causes for the changes if the research design is restricted enough. Using both microscopic techniques and chemical analysis, it may be easier to understand the ensiling process. This in turn would ultimately lead to improving silage as a feedstuff for livestock, which is one of the initial goals of any silage research.

Since this research was exploratory, in the fact that silage had not been studied before microscopically, the methods used in this study did not restrict the areas of observations except for the sampling dates and times. As a general study it covered a lot of area and indicated that further research was warranted and needed in order to answer the questions left unanswered by this study.

Results of this research illustrated five major ultrastructural changes. In the silage sections the cell contents were disrupted

and some decomposition occurred during ensiling. Most of the cell walls were found intact but some breakage and erosion occurred. Starch was found present within the chloroplasts of fresh tissue, and when present in silage sections starch was seen among disrupted cytoplasm. Differences were noted in the quantities of starch produced in fresh leaves and stems; leaves having a much larger production of starch than stems. Lipid was visible as coalesced droplets in silage sections, but was rarely coalesced in fresh sections.

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Appendix 1

Preparation of 2.5% Glutaraldehyde Solution

A phosphate buffer was made using 2.652 g of KH_2PO_4 (crystal form) and 5.307 g of K_2HPO_4 (powder form). These two compounds were added to a 1000 ml volumetric flask and glass distilled water was added until the volume mark was reached. The pH was checked and determined as 7.13. Different fixation procedures specify different pH ranges. The buffer was stored at 3 C.

After the phosphate buffer was made, it was used to make up the 2.5% glutaraldehyde solution when needed. Just prior to sampling, a fresh solution of glutaraldahyde was made by adding 5 ml of 3 C, 50% glutaraldehyde solution to 95 ml of 3 C premixed phosphate buffer. The 2.5 glutaraldehyde solution remaining after fixation was discarded.

Appendix 2

Preparation of 1% Osmium Tetroxide

Ampules containing 0.5 g of osmium tetroxide (OsO_4) were used. An ampule was first scratched with a diamond pencil and then placed in a small necked flask and broken by a glass stirring rod. Then 50 ml of glass distilled water was added to the flask. All of the above procedures, including the mixing, were done under a hood while wearing safety glasses. After mixing, the solution was ready for use. The osmium tetroxide ampules and 1% solution were stored at 3 C.

Appendix 3

Preparation of Spurr Plastic

A plastic 200 ml cup was weighed on a top loading balance. Then specific amounts of the component plastic chemicals were added to the cup. All the chemicals, being in liquid form, were added by pasteur pipettes in the following order: 20 g of vinylcyclohexene dioxide (VCD), 12 g of diglycidyl ether of polypropyleneglycol (DER 736), 52 g of nonenyl succinic anhydride (NSA), and 0.8 g of dimethylaminoethanol (DAE). The NSA was stored at 3 C until needed, then allowed to reach room temperature before use. The remaining chemicals were stored at room temperature. Once measured, the four plastics were stirred for 15 min and then used as needed. All unused plastic was discarded after 4 h.

Appendix 4

Preparation of 2% Uranyl Acetate

One half gram of uranyl acetate crystals were weighed on a piece of weighing paper. The uranyl acetate crystals were then added to the 25 ml volumetric flask, and the flask was partially filled with glass distilled water. After the uranyl acetate crystals were dissolved, more water was added to bring the level up to volume. The solution was then mixed again and stored in a dark area. After 48 h the solution was used with care, so as not to disturb the residue which had formed on the flask's bottom. the uranyl acetate solution was always stored in the dark, except during use.

Appendix 5

Preparation of 0.5% Lead Citrate

Glass distilled water was boiled and then cooled. A 100 ml volumetric flask was then partially filled with the boiled water. One half gram of lead citrate was added to the flask. Eight ml of 2 N, CO_2 free, NaOH was added next. The solution was mixed until clear. Then more of the boiled water was added to bring the flask to volume. After a second mixing, aliquots were placed in snap cap vials and a layer of mineral oil was used to cover the solution. The vials were stored, with caps in place, at 3 C. When needed for use the vial was first warmed to room temperature, then used.